# WEST Search History

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DATE: Wednesday, April 27, 2005

| Hide? | Set Name | Query | Hit Count |
|---|---|---|---|
| | *DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD; PLUR=YES; OP=AND* | | |
| ☐ | L1 | (george or angelos or hess).in. | 291380 |
| ☐ | L2 | L1 and moraxel$ | 31 |
| ☐ | L3 | l2 and ($toxin or toxi$) | 18 |
| ☐ | L4 | l3 and bovi$ | 13 |
| ☐ | L5 | moraxella.clm. and bovis!.clm. | 42 |
| ☐ | L6 | L5 not l4 | 41 |
| ☐ | L7 | L6 and ($toxin or toxi$).clm. | 7 |

END OF SEARCH HISTORY

1. 20040265326. 29 Jul 04. 30 Dec 04. Subunit respiratory syncytial virus vaccine preparation. Cates, George A., et al. 424/186.1; 435/320.1 435/325 435/456 435/69.3 530/350 536/23.7 C07H021/04 A61K039/12 C07K014/005 C12N015/86.

2. 20040147474. 09 Jan 04. 29 Jul 04. Novel compounds. Ruelle, Jean-Louis, et al. 514/44; 435/320.1 435/325 435/6 435/69.1 530/350 536/23.2 A61K048/00 C12Q001/68 C07H021/04.

3. 20040131625. 03 Feb 04. 08 Jul 04. Vaccine composition. Berthet, Francois-Xavier Jacques, et al. 424/184.1; A61K039/00 A61K039/38 A61K039/118.

4. 20040126389. 15 Sep 03. 01 Jul 04. Vaccine composition. Berthet, Francois-Xavier Jacques, et al. 424/190.1; 424/236.1 424/244.1 424/256.1 514/54 A61K039/09 A61K039/102 A61K031/739.

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10. 20020119573. 28 Feb 01. 29 Aug 02. Footprinting plasmid. Shaw, Karen J., et al. 435/473; 435/252.3 435/320.1 435/6 C12N015/74 C12Q001/68 C12N001/21.

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14. 6706270. 06 Sep 01; 16 Mar 04. Compounds. Ruelle; Jean-Louis, et al. 424/250.1; 424/184.1 424/185.1 424/190.1 424/249.1 435/243 435/252.3 435/69.1 435/69.3 530/300 530/350 536/23.1

536/23.7 536/24.1 536/24.32. A61K039/05 C12P021/06 C12N015/09 C12N001/20.

☐ 15. 6610478. 15 Aug 97; 26 Aug 03. Phenotypic conversion of cells mediated by external guide sequences. Takle; Garry B., et al. 435/6; 435/471 435/69.1 540/145. C12Q001/68 C12P021/06 C12N015/74 C07B047/00.

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☐ 25. 5776912. 20 Dec 96; 07 Jul 98. Lipophilic oligosaccharide antibiotic compositions. Patel; Mahesh G., et al. 514/54; 536/123.1 536/16.8. A61K031/70.

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CYTOTOXIN GENE, ANTIBODIES AND VACCINES FOR PREVENTION AND TREATMENT OF MORAXELLA BOVIS INFECTIONS. GEORGE, LISLE W, et al. A61K039/02; C07K014/22.

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30. WO2002102408A. New Moraxella bovis cytotoxins and cytotoxin genes, useful as vaccines for preventing or treating infectious bovine keratoconjunctivitis (pinkeye) caused by M. bovis. ANGELOS, J A, et al. A61K039/02 C07K014/22.

31. WO 9007525A. Keratoconjunctivitis cytotoxin from Moraxella bovis - toxic to bovine peripheral blood neutrophils but lacks haemolytic activity. GEORGE, L W, et al. A61K037/48 A61K039/02 C07K015/00.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00106

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 15/00, 15/02, 15/04; A61K 39/02, 37/48
U.S. CL.: 530/350; 424/88,92

## II. FIELDS SEARCHED

Minimum Documentation Searched 7

| Classification System | Classification Symbols |
|---|---|
| U.S. CL. | 530/350<br>424/88,92 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Data bases: Chemical Abstract Services ONline (1967-1990), File CA; File Biosis). Automated Patent Searching (1975-1990). Search Terms: Moraxella bovis, Toxin Cytotxin

## III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

| Category * | Citation of Document, 11 with indication, where appropriate, of the relevant passages 12 | Relevant to Claim No. 13 |
|---|---|---|
| X T<br>Y | American Journal of Veterinary Research, Volume 51(2), issued February 1990, Hoien-Dalen et al.,"Comparative Characterization of the Leukocidic and Hemolytic Activity of Moraxella bocis ," pages 191-196. See entire document. | 1-13<br>1-13 |
| X P<br>Y | American Journal of Veterinary Research, Volume 50(1), issued January 1989, Kagonyera et al. "Effects of Moraxella bovis and Culture filtrates on $^{51}$Cr-labeled Bovine Neutrophils,"pages 18-21. See the Disussion pages 20-21. | 1-5<br>1-13 |
| X<br>Y | Conference of Research Works in Animal Discase, Abstract Number 49, Published 1988, Hoicn-Dalen et al., "Partial Characterization of a Moraxella bovis Leukocidin and Comparison to the M. bovis Hemolysin, "page 9. See the entire abstract. | 1-5<br>1-13 |
| Y | Canadian Journal of Comparative Medicine, Volume 37, issued January 1973, Pugh et al., "The Pathophysiological Effects of Moraxella bovis Toxins on Cattle, Mice, an Guinea Pigs, "pages 70-78. See the Abstract and pages 76-77. | 1-13 |

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 02 April 1990 | 03 MAY 1990 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| ISA/US | R. Keith Baker |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| Y | American Journal of Veterinary Research, Volume 36(3), Issued March 1975, Hughes et al., "Experimentally Induced Infectious Bovine Keratoconjunctivitis; Relationship of Vaccination Schedule to Protection Against Exposure with Homologous Moraella bouis Culture," pages 263-265. See the Summary and the last paragraph. | 6-13 |
| Y | WO,A 86/06635 (Biotechnology Australia Pty, LTD.) 20 November 1986 (20.11.86) See the Abstract claims 12 and 13. | 6-13 |

L7: Entry 5 of 7 File: USPT Aug 15, 2000

DOCUMENT-IDENTIFIER: US 6103243 A
TITLE: Oral vaccines

CLAIMS:

2. A protein conjugate according to claim 1, wherein said carrier molecule is heat labile toxin of enterotoxigenic E. coli.

3. A protein conjugate according to claim 1, wherein said carrier molecule is the binding subunit of heat labile toxin of enterotoxigenic E. coli.

11. A protein conjugate according to claim 1, wherein said immunogen is a pilus from an organism selected from the group consisting of E. coli, N. gonorrheae, N, neningitidis, N. catarrhalis, yersinia, pseudomonas, moraxella bovis, bacteroides nodosus, staphylococcus, streptococcus, and bordetella.

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 5 : | | (11) International Publication Number: WO 90/07525 |
|---|---|---|
| C07K 15/00, 15/02, 15/04<br>A61K 39/02, 37/48 | A1 | (43) International Publication Date: 12 July 1990 (12.07.90) |

(21) International Application Number: PCT/US90/00106

(22) International Filing Date: 5 January 1990 (05.01.90)

(30) Priority data:
294,239 6 January 1989 (06.01.89) US

(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, 22nd Floor, Oakland, CA 94612 (US).

(72) Inventors: GEORGE, Lisle, W. ; 2906 Temple Drive, Davis, CA 95616 (US). KAGONYERA, George, M. ; P.O. Box 7003, Kampala (UG).

(74) Agent: WEBER, Ellen, Lauver; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).

(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: KERATOCONJUNCTIVITIS CYTOTOXIN AND METHODS FOR ITS USE AND PRODUCTION

(57) Abstract

This invention relates to a cytotoxin from *Moraxella bovis* substantially free of intact microbial cells, which is toxic to bovine peripheral blood neutrophils and which lacks hemolytic activity. This cytotoxin is useful as a vaccine for protection of animals from infectious bovine keratoconjunctivitis. The cytotoxin is further characterized in that it is not capable of hydrolyzing casein, it is net negatively charged at a pH of 7.4 and by its ability to elute off of a DEAE exchange column with a 3ml gel bed under a salt gradient at a 0.2M to about a 0.3M salt concentration. Further, the cytotoxic activity to bovine peripheral blood neutrophils is sensitive to zinc salts. Typically, the cytotoxin is prepared by clarifying and purifying culture filtrates of *Moraxella bovis*.

# UniProtKB/TrEMBL entry Q93GI2



*Note: most headings are clickable, even if they don't appear as links. They link to the user manual or other documents.*

## Entry information

| | |
|---|---|
| Entry name | **Q93GI2_MORBO** |
| Primary accession number | **Q93GI2** |
| Secondary accession numbers | None |
| Entered in TrEMBL in | Release 19, December 2001 |
| Sequence was last modified in | Release 19, December 2001 |
| Annotations were last modified in | Release 26, March 2004 |

updated search

## Name and origin of the protein

| | |
|---|---|
| Protein name | **RTX toxin** |
| Synonyms | None |
| Gene name | **Name: mbxA** |
| From | Moraxella bovis [TaxID: 476] |
| Taxonomy | Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Moraxella. |

## References

[1] NUCLEOTIDE SEQUENCE.
**STRAIN**=Tifton I;
MEDLINE=21388402;PubMed=11497442 [NCBI, ExPASy, EBI, Israel, Japan]
Angelos J.A., Hess J.F., George L.W.;
"Cloning and characterization of a Moraxella bovis cytotoxin gene.";
Am. J. Vet. Res. 62:1222-1228(2001).

[2] NUCLEOTIDE SEQUENCE.
**STRAIN**=Tifton I;
Angelos J.A., Hess J.F., George L.W.;
Submitted (DEC-2002) to the EMBL/GenBank/DDBJ databases.

## Comments

- ***SUBCELLULAR LOCATION***: Secreted *(By similarity)*.
- ***DOMAIN***: The Gly-rich region is probably involved in binding calcium, which is required for target cell-binding or cytolytic activity *(By similarity)*.

## Cross-references

| | | |
|---|---|---|
| EMBL | AF205359; AAK84651.1; -.[EMBL / GenBank / DDBJ] [CoDingSequence] | |
| | GO:0005576; | Cellular component: extracellular region *(inferred from electronic annotation)*. |
| GO | GO:0005509; | Molecular function: calcium ion binding *(inferred from electronic annotation)*. |

| | |
|---|---|
| | GO:0019835; Biological process: cytolysis *(inferred from electronic annotation)*.<br>GO:0009405; Biological process: pathogenesis *(inferred from electronic annotation)*.<br>QuickGo<br>view. |
| InterPro | IPR002048; EF-hand.<br>IPR001343; Hemlysn_Ca_bind.<br>IPR003995; RtxA.<br>Graphical view of domain structure. |
| Pfam | PF00353; HemolysinCabind; 5.<br>PF02382; RTX; 1.<br>Pfam graphical view of domain structure. |
| PRINTS | PR00313; CABNDNGRPT.<br>PR01488; RTXTOXINA. |
| PROSITE | PS00018; EF_HAND; UNKNOWN_1.<br>PS00330; HEMOLYSIN_CALCIUM; 2. |
| ProDom | [Domain structure / List of seq. sharing at least 1 domain] |
| HOGENOM | [Family / Alignment / Tree] |
| ProtoMap | Q93GI2. |
| PRESAGE | Q93GI2. |
| ModBase | Q93GI2. |
| SWISS-2DPAGE | Get region on 2D PAGE. |
| UniRef | View cluster of proteins with at least 50% / 90% identity. |

## Keywords

**Calcium**; **Cytolysis**; **Repeat**; **Toxin**.

## Features

None

## Sequence information

Length: **927 AA** Molecular weight: **98845 Da** CRC64: **F4B703577E10A96D** [This is a checksum on the sequence]

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        10         20         30         40         50         60
MSNINVIKSN IQAGLNSTKS GLKNLYLAIP KDYDPQKGGT LNDFIKAADE LGIARLAEEP

        70         80         90        100        110        120
NHTETAKKSV DTVNQFLSLT QTGIAISATK LEKFLQKHST NKLAKGLDSV ENIDRKLGKA

       130        140        150        160        170        180
SNVLSTLSSF LGTALAGIEL DSLIKKGDAA PDALAKASID LINEIIGNLS QSTQTIEAFS

       190        200        210        220        230        240
SQLAKLGSTI SQAKGFSNIG NKLQNLNFSK TNLGLEIITG LLSGISAGFA LADKNASTGK

       250        260        270        280        290        300
KVAAGFELSN QVIGNVTKAI SSYVLAQRVA AGLSTTGAVA ALITSSIMLA ISPLAFMNAA

       310        320        330        340        350        360
DKFNHANALD EFAKQFRKFG YDGDHLLAEY QRGVGTIEAS LTTISTALGA VSAGVSAAAV

       370        380        390        400        410        420
GSAVGAPIAL LVAGVTGLIS GILEASKQAM FESVANRLQG KILEWEKQNG GQNYFDKGYD
```

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       430        440        450        460        470        480
SRYAAYLANN LKFLSELNKE LEAERVIAIT QQRWDNNIGE LAGITKLGER IKSGKAYADA

       490        500        510        520        530        540
FEDGKKVEAG SNITLDAKTG IIDISNSNGK KTQALHFTSP LLTAGTESRE RLTNGKYSYI

       550        560        570        580        590        600
NKLKFGRVKN WQVTDGEASS KLDFSKVIQR VAETEGTDEI GLIVNAKAGN DDIFVGQGKM

       610        620        630        640        650        660
NIDGGDGHDR VFYSKDGGFG NITVDGTSAT EAGSYTVNRK VARGDIYHEV VKRQETKVGK

       670        680        690        700        710        720
RTETIQYRDY ELRKVGYGYQ STDNLKSVEE VIGSQFNDVF KGSKFNDIFH SGEGDDLLDG

       730        740        750        760        770        780
GAGDDRLFGG KGNDRLSGDE GDDLLDGGSG DDVLNGGAGN DVYIFRKGDG NDTLYDGTGN

       790        800        810        820        830        840
DKLAFADANI SDIMIERTKE GIIVKRNDHS GSINIPRWYI TSNLQNYQSN KTDHKIEQLI

       850        860        870        880        890        900
GKDGSYITSD QIDKILQDKK DGTVITSQEL KKLADENKSQ KLSASDIASS LNKLVGSMAL

       910        920
FGTANSVSSN ALQPITQPTQ GILAPSV
```

Q93GI2 in FASTA format

*View entry in original TrEMBL format*
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*Request for annotation of this TrEMBL entry*

BLAST submission on ExPASy/SIB or at NCBI (USA)

Sequence analysis tools: ProtParam, ProtScale, Compute pI/Mw, PeptideMass, PeptideCutter, Dotlet (Java)

ScanProsite, MotifScan

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==========

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If results of this search are reported or published, please mention that the computation was performed at the SIB using the BLAST network service. The SIB BLAST network service uses a server developed at SIB and the NCBI BLAST 2 software.

In case of problems, please read the online BLAST help.
If your question is not covered, please contact <helpdesk@expasy.org>.

NCBI BLAST program reference [PMID:9254694]:
Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402(1997).

==========

Query: 12 AA
Date run: 2005-04-27 12:57:23 UTC+0100 on sib-gm1.unil.ch
Program: NCBI BLASTP 1.5.4-Paracel [2003-06-05]
Database: EXPASY/UniProt
1,880,849 sequences; 604,459,357 total letters
UniProt Release 4.6 consists of: Swiss-Prot Release 46.6 of 26-Apr-2005: 180652 en
TrEMBL Release 29.6 of 26-Apr-2005: 1689375 entrie

Taxonomic view | NiceBlast view | Printable view

**List of potentially matching sequences**

Send selected sequences to Clustal W (multiple alignment) Submit Query
Select up to...

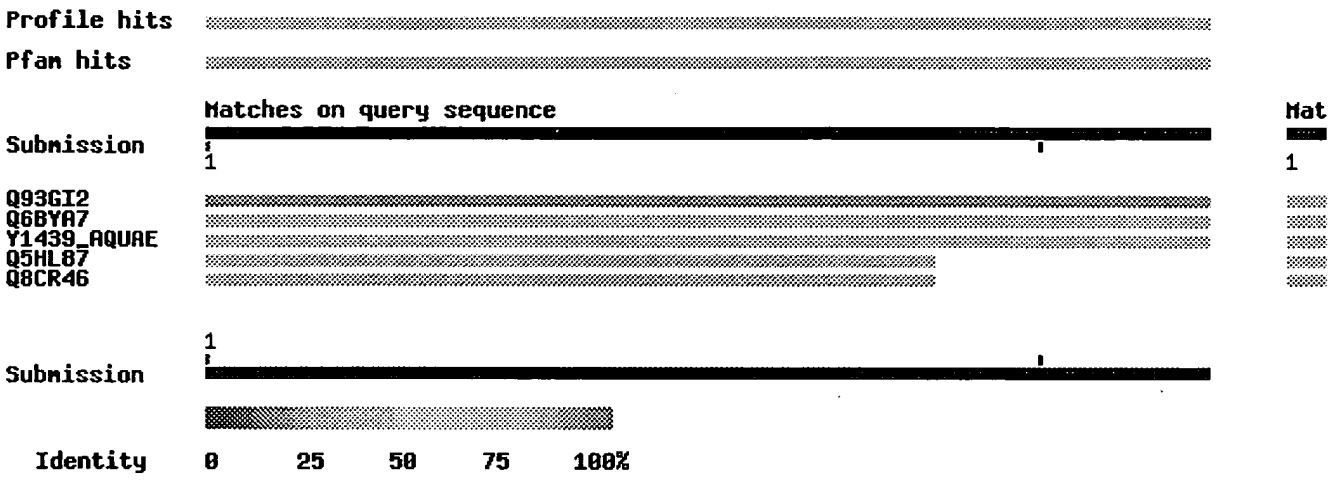
☐ Include query sequence

| | Db | AC | Description | Score | E-value |
|---|---|---|---|---|---|
| ☐ | tr | Q93GI2 | _MORBO RTX toxin [mbxA] [Moraxella bovis] | 41 | 0.003 |
| ☐ | tr | Q6BYA7 | _DEBHA Similarity [DEHA0A11517g] [Debaryomyces hansenii... | 31 | 3.0 |
| ☐ | sp | O67428 | Y1439_AQUAE Hypothetical protein AQ_1439 precursor [AQ... | 29 | 9.6 |
| ☐ | tr | Q5HL87 | _STAEQ Ribokinase (EC 2.7.1.15) [rbsK] [Staphylococcus ... | 29 | 9.6 |
| ☐ | tr | Q8CR46 | _STAEP Ribokinase [SE2086] [Staphylococcus epidermidis] | 29 | 9.6 |

**Graphical overview of the alignments**

Click here to resubmit your query after masking regions matching PROSITE profiles or Pfam HMMs

(Help) (use ScanProsite for more details about PROSITE matches)




**Alignments**

tr Q93GI2 Q93GI2_MORBO **RTX toxin [mbxA] [Moraxella bovis]** 927 AA

align

Score = 40.9 bits (89), Expect = 0.003
Identities = 12/12 (100%), Positives = 12/12 (100%)

```
Query: 1   FLSELNKELEAE 12
           FLSELNKELEAE
Sbjct: 433 FLSELNKELEAE 444
```

tr Q6BYA7 Q6BYA7_DEBHA **Similarity [DEHA0A11517g] [Debaryomyces hansenii (Yeast) (Torulaspora hansenii)]** 387 AA

align

Score = 30.8 bits (65), Expect = 3.0
Identities = 10/12 (83%), Positives = 10/12 (83%)

```
Query: 1   FLSELNKELEAE 12
           FLSELNK EAE
Sbjct: 123 FLSELNKNYEAE 134
```

sp Q67428 Y1439_AQUAE **Hypothetical protein AQ_1439 precursor [AQ_1439] [Aquifex aeolicus]** 617 AA

align

Score = 29.1 bits (61), Expect = 9.6
Identities = 10/12 (83%), Positives = 10/12 (83%), Gaps = 1/12 (8%)

```
Query: 1   FLSELNKELEAE 12
```

```
            FLSE  KELEAE
Sbjct: 443 FLSE-GKELEAE 453
```

tr Q5HL87 Q5HL87_STAEQ **Ribokinase (EC 2.7.1.15) [rbsK] [Staphylococcus epidermidis (strain ATCC 35984 / RP62A)]** 307 AA align

```
 Score = 29.1 bits (61), Expect = 9.6
 Identities = 8/9 (88%), Positives = 9/9 (99%)

Query: 1   FLSELNKEL 9
           FLSELNK+L
Sbjct: 261 FLSELNKDL 269
```

tr Q8CR46 Q8CR46_STAEP **Ribokinase [SE2086] [Staphylococcus epidermidis]** 307 AA align

```
 Score = 29.1 bits (61), Expect = 9.6
 Identities = 8/9 (88%), Positives = 9/9 (99%)

Query: 1   FLSELNKEL 9
           FLSELNK+L
Sbjct: 261 FLSELNKDL 269
```

```
Database: EXPASY/UniProt
    Posted date:  Apr 25, 2005  4:19 PM
  Number of letters in database: 604,459,357
  Number of sequences in database:  1,880,849

Lambda     K      H
   0.319    0.287     1.52

Gapped
Lambda     K      H
   0.294    0.110    0.610


Matrix: PAM30
Gap Penalties: Existence: 9, Extension: 1
length of query: 12
length of database: 604,459,357
effective HSP length: 3
effective length of query: 9
effective length of database: 598,816,810
effective search space: 5389351290
effective search space used: 5389351290
T: 16
A: 40
X1: 16 ( 7.4 bits)
X2: 35 (14.8 bits)
X3: 58 (24.6 bits)
```

```
S1: 43 (21.6 bits)
S2: 61 (29.1 bits)

Wallclock time: 2 seconds
```

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==========

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NCBI BLAST program reference [PMID:9254694]:
Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402(1997).

==========

Query: 14 AA
Date run: 2005-04-27 12:58:46 UTC+0100 on sib-gm1.unil.ch
Program: NCBI BLASTP 1.5.4-Paracel [2003-06-05]
Database: EXPASY/UniProt
1,880,849 sequences; 604,459,357 total letters
UniProt Release 4.6 consists of: Swiss-Prot Release 46.6 of 26-Apr-2005: 180652 en
TrEMBL Release 29.6 of 26-Apr-2005: 1689375 entrie

Taxonomic view | NiceBlast view | Printable view

**List of potentially matching sequences**

Send selected sequences to Clustal W (multiple alignment) Submit Query
Select up to...

☐ Include query sequence

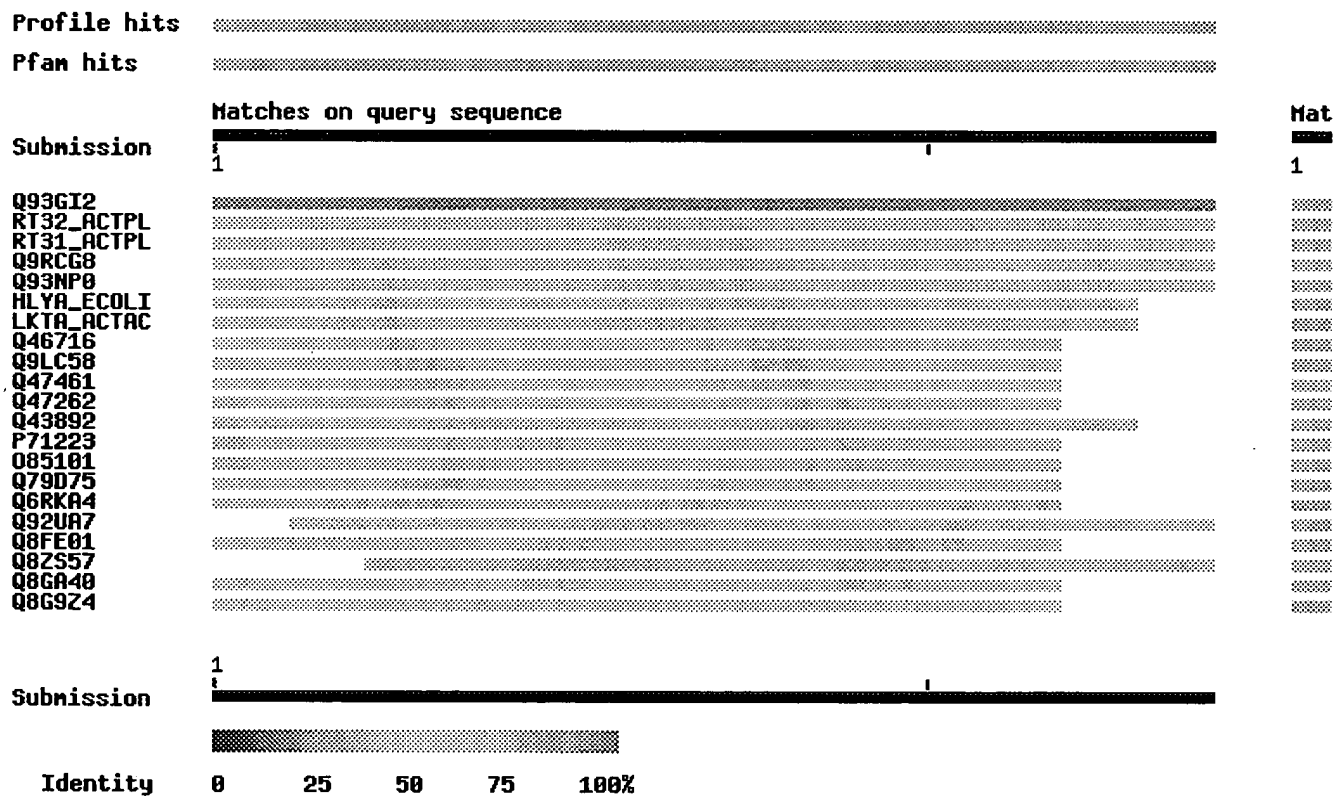
| | Db | AC | Description | Score | E-value |
|---|---|---|---|---|---|
| ☐ | tr | Q93GI2 | _MORBO RTX toxin [mbxA] [Moraxella bovis] | 49 | 1e-05 |
| ☐ | sp | P55131 | RT32_ACTPL RTX-III toxin determinant A from serotype 8... | 33 | 0.51 |
| ☐ | sp | P55130 | RT31_ACTPL RTX-III toxin determinant A from serotype 2... | 33 | 0.51 |
| ☐ | tr | Q9RCG8 | _PASAE PaxA [paxA] [Pasteurella aerogenes] | 33 | 0.51 |
| ☐ | tr | Q93NP0 | _ACTPL RTX-toxin IIIA [Actinobacillus pleuropneumoniae ... | 33 | 0.51 |
| ☐ | sp | P08715 | HLYA_ECOLI Hemolysin, plasmid [hlyA] [Escherichia coli] | 32 | 1.2 |
| ☐ | sp | P16462 | LKTA_ACTAC Leukotoxin [lktA] [Actinobacillus actinomyc... | 31 | 3.0 |
| ☐ | tr | Q46716 | _ECO57 Hemolysin A (Hemolysin toxin protein) [hlyA] [Es... | 31 | 3.0 |
| ☐ | tr | Q9LC58 | _ECOLI Hemolysin A [EHEC-hlyA] [Escherichia coli] | 31 | 3.0 |
| ☐ | tr | Q47461 | _ECOLI EHEC-hlyA protein [EHEC-hlyA] [Escherichia coli] | 31 | 3.0 |

| | | | | |
|---|---|---|---|---|
| ☐ | tr Q47262 | _ECOLI Hemolysin [EHEC-hlyA] [Escherichia coli] | 31 | 3.0 |
| ☐ | tr Q43892 | _ACTAC Leukotoxin [LKTA] [Actinobacillus actinomycetemc... | 31 | 3.0 |
| ☐ | tr P71223 | _ECOLI EHEC-hemolysin [EHEC-hlyA] [Escherichia coli] | 31 | 3.0 |
| ☐ | tr O85101 | _ECOLI Hemolysin [ehxA] [Escherichia coli] | 31 | 3.0 |
| ☐ | tr Q79D75 | _ECOLI HlyA (Fragment) [hlyA] [Escherichia coli] | 31 | 3.0 |
| ☐ | tr Q6RKA4 | _ECOLI HlyA (Fragment) [Escherichia coli] | 31 | 3.0 |
| ☐ | tr Q92UA7 | _RHIME Hypothetical calcium binding protein [SMb21402] ... | 29 | 7.1 |
| ☐ | tr Q8FE01 | _ECOL6 Hemolysin A [hlyA] [Escherichia coli O6] | 29 | 9.6 |
| ☐ | tr Q8ZS57 | _ANASP All7655 protein [all7655] [Anabaena sp. (strain ... | 29 | 9.6 |
| ☐ | tr Q8GA40 | _ECOLI Hemolysin A [hlyA] [Escherichia coli] | 29 | 9.6 |
| ☐ | tr Q8G9Z4 | _ECOLI HlyA protein [hlyA] [Escherichia coli] | 29 | 9.6 |

**Graphical overview of the alignments**

Click here to resubmit your query after masking regions matching PROSITE profiles or Pfam HMMs

(? Help) (use ScanProsite for more details about PROSITE matches)




**Alignments**

tr Q93GI2 Q93GI2_MORBO **RTX toxin [mbxA] [Moraxella bovis]** 927 AA

align

Score = 48.6 bits (107), Expect = 1e-05
Identities = 14/14 (100%), Positives = 14/14 (100%)

```
Query: 1   FNDIFHSGEGDDLL 14
           FNDIFHSGEGDDLL
Sbjct: 705 FNDIFHSGEGDDLL 718
```

sp P55131 RT32_ACTPL **RTX-III toxin determinant A from serotype 8 (APX-IIIA) (Cytolysin IIIA) (CLY-IIIA) [apxIIIA] [Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)]** 1052 AA align

Score = 33.3 bits (71), Expect = 0.51
Identities = 10/14 (71%), Positives = 11/14 (78%)

```
Query: 1   FNDIFHSGEGDDLL 14
           F DIFH  +GDDLL
Sbjct: 748 FRDIFHGADGDDLL 761
```

sp P55130 RT31_ACTPL **RTX-III toxin determinant A from serotype 2 (APX-IIIA) (Cytolysin IIIA) (CLY-IIIA) [apxIIIA] [Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)]** 1049 AA align

Score = 33.3 bits (71), Expect = 0.51
Identities = 10/14 (71%), Positives = 11/14 (78%)

```
Query: 1   FNDIFHSGEGDDLL 14
           F DIFH  +GDDLL
Sbjct: 747 FRDIFHGADGDDLL 760
```

tr Q9RCG8 Q9RCG8_PASAE **PaxA [paxA] [Pasteurella aerogenes]** 1049 AA align

Score = 33.3 bits (71), Expect = 0.51
Identities = 10/14 (71%), Positives = 11/14 (78%)

```
Query: 1   FNDIFHSGEGDDLL 14
           F DIFH  +GDDLL
Sbjct: 748 FRDIFHGADGDDLL 761
```

tr Q93NP0 Q93NP0_ACTPL **RTX-toxin IIIA [Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)]** 1052 AA align

Score = 33.3 bits (71), Expect = 0.51

Identities = 10/14 (71%), Positives = 11/14 (78%)

```
Query: 1   FNDIFHSGEGDDLL 14
           F DIFH  +GDDLL
Sbjct: 748 FRDIFHGADGDDLL 761
```

sp P08715 HLYA_ECOLI **Hemolysin, plasmid [hlyA] [Escherichia coli]** 1024 AA

align

Score = 32.0 bits (68), Expect = 1.2
Identities = 9/13 (69%), Positives = 10/13 (76%)

```
Query: 1   FNDIFHSGEGDDL 13
           F DIFH  +GDDL
Sbjct: 736 FTDIFHGADGDDL 748
```

sp P16462 LKTA_ACTAC **Leukotoxin [lktA] [Actinobacillus actinomycetemcomitans (Haemophilus actinomycetemcomitans)]** 1050 AA

align

Score = 30.8 bits (65), Expect = 3.0
Identities = 9/13 (69%), Positives = 11/13 (84%)

```
Query: 1   FNDIFHSGEGDDL 13
           FND+FH  +GDDL
Sbjct: 734 FNDVFHGHDGDDL 746
```

tr Q46716 Q46716_ECO57 **Hemolysin A (Hemolysin toxin protein) [hlyA] [Escherichia coli O157:H7]** 998 AA

align

Score = 30.8 bits (65), Expect = 3.0
Identities = 8/12 (66%), Positives = 10/12 (82%)

```
Query: 1   FNDIFHSGEGDD 12
           FNDIFH  +G+D
Sbjct: 720 FNDIFHGADGND 731
```

tr Q9LC58 Q9LC58_ECOLI **Hemolysin A [EHEC-hlyA] [Escherichia coli]** 998 AA

align

Score = 30.8 bits (65), Expect = 3.0
Identities = 8/12 (66%), Positives = 10/12 (82%)

```
Query: 1   FNDIFHSGEGDD 12
```

```
           FNDIFH  +G+D
Sbjct: 720 FNDIFHGADGND 731
```

tr Q47461 **EHEC-hlyA protein [EHEC-hlyA] [Escherichia coli]** 998 AA
Q47461_ECOLI

align

```
 Score = 30.8 bits (65), Expect = 3.0
 Identities = 8/12 (66%), Positives = 10/12 (82%)

Query: 1   FNDIFHSGEGDD 12
           FNDIFH  +G+D
Sbjct: 720 FNDIFHGADGND 731
```

tr Q47262 **Hemolysin [EHEC-hlyA] [Escherichia coli]** 998 AA
Q47262_ECOLI

align

```
 Score = 30.8 bits (65), Expect = 3.0
 Identities = 8/12 (66%), Positives = 10/12 (82%)

Query: 1   FNDIFHSGEGDD 12
           FNDIFH  +G+D
Sbjct: 720 FNDIFHGADGND 731
```

tr Q43892 **Leukotoxin [LKTA] [Actinobacillus actinomycetemcomitans** 1055 AA
Q43892_ACTAC **(Haemophilus actinomycetemcomitans)]**

align

```
 Score = 30.8 bits (65), Expect = 3.0
 Identities = 9/13 (69%), Positives = 11/13 (84%)

Query: 1   FNDIFHSGEGDDL 13
           FND+FH  +GDDL
Sbjct: 734 FNDVFHGHDGDDL 746
```

tr P71223 **EHEC-hemolysin [EHEC-hlyA] [Escherichia coli]** 998 AA
P71223_ECOLI

align

```
 Score = 30.8 bits (65), Expect = 3.0
 Identities = 8/12 (66%), Positives = 10/12 (82%)

Query: 1   FNDIFHSGEGDD 12
           FNDIFH  +G+D
Sbjct: 720 FNDIFHGADGND 731
```

tr Q85101 **Hemolysin [ehxA] [Escherichia coli]** 998 AA
Q85101_ECOLI
align

Score = 30.8 bits (65), Expect = 3.0
Identities = 8/12 (66%), Positives = 10/12 (82%)

```
Query: 1   FNDIFHSGEGDD 12
           FNDIFH  +G+D
Sbjct: 720 FNDIFHGADGND 731
```

tr Q79D75 **HlyA (Fragment) [hlyA] [Escherichia coli]** 758 AA
Q79D75_ECOLI
align

Score = 30.8 bits (65), Expect = 3.0
Identities = 8/12 (66%), Positives = 10/12 (82%)

```
Query: 1   FNDIFHSGEGDD 12
           FNDIFH  +G+D
Sbjct: 480 FNDIFHGADGND 491
```

tr Q6RKA4 **HlyA (Fragment) [Escherichia coli]** 308 AA
Q6RKA4_ECOLI
align

Score = 30.8 bits (65), Expect = 3.0
Identities = 8/12 (66%), Positives = 10/12 (82%)

```
Query: 1   FNDIFHSGEGDD 12
           FNDIFH  +G+D
Sbjct: 224 FNDIFHGADGND 235
```

tr Q92UA7 **Hypothetical calcium binding protein [SMb21402]** 387
Q92UA7_RHIME **[Rhizobium** AA
**meliloti (Sinorhizobium meliloti)]** align

Score = 29.5 bits (62), Expect = 7.1
Identities = 9/13 (69%), Positives = 10/13 (76%)

```
Query: 2  NDIFHSGEGDDLL 14
          NDIF  GEG+D L
Sbjct: 81 NDIFDGGEGNDVL 93
```

tr Q8FE01 **Hemolysin A [hlyA] [Escherichia coli O6]** 1024 AA
Q8FE01_ECOL6

align

Score = 29.1 bits (61), Expect = 9.6
Identities = 8/12 (66%), Positives = 9/12 (74%)

```
Query: 1   FNDIFHSGEGDD 12
           F DIFH  +GDD
Sbjct: 736 FTDIFHGADGDD 747
```

tr Q8ZS57 Q8ZS57_ANASP **All7655 protein [all7655] [Anabaena sp. (strain PCC 7120)]** 302 AA
align

Score = 29.1 bits (61), Expect = 9.6
Identities = 9/12 (75%), Positives = 9/12 (75%)

```
Query: 3  DIFHSGEGDDLL 14
          DIFH G  DDLL
Sbjct: 85 DIFHRGGADDLL 96
```

tr Q8GA40 Q8GA40_ECOLI **Hemolysin A [hlyA] [Escherichia coli]** 1024 AA
align

Score = 29.1 bits (61), Expect = 9.6
Identities = 8/12 (66%), Positives = 9/12 (74%)

```
Query: 1   FNDIFHSGEGDD 12
           F DIFH  +GDD
Sbjct: 736 FTDIFHGADGDD 747
```

tr Q8G9Z4 Q8G9Z4_ECOLI **HlyA protein [hlyA] [Escherichia coli]** 1024 AA
align

Score = 29.1 bits (61), Expect = 9.6
Identities = 8/12 (66%), Positives = 9/12 (74%)

```
Query: 1   FNDIFHSGEGDD 12
           F DIFH  +GDD
Sbjct: 736 FTDIFHGADGDD 747
```

Database: EXPASY/UniProt
Posted date: Apr 25, 2005 4:19 PM
Number of letters in database: 604,459,357
Number of sequences in database: 1,880,849

| Lambda | K | H |
|---|---|---|
| 0.335 | 0.294 | 1.73 |

```
Gapped
Lambda      K       H
   0.294    0.110    0.610


Matrix: PAM30
Gap Penalties: Existence: 9, Extension: 1
Number of HSP's successfully gapped in prelim test: 0
length of query: 14
length of database: 604,459,357
effective HSP length: 5
effective length of query: 9
effective length of database: 595,055,112
effective search space: 5355496008
effective search space used: 5355496008
T: 16
A: 40
X1: 15 ( 7.2 bits)
X2: 35 (14.8 bits)
X3: 58 (24.6 bits)
S1: 41 (21.6 bits)
S2: 61 (29.1 bits)

Wallclock time: 2 seconds
```

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# WEST Search History

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DATE: Wednesday, April 27, 2005

| Hide? | Set Name | Query | Hit Count |
|---|---|---|---|
| | *DB=PGPB; PLUR=YES; OP=AND* | | |
| ☐ | L1 | 20030035809 | 1 |
| ☐ | L2 | L1 and 18 | 1 |
| ☐ | L3 | L1 and 32 | 1 |
| ☐ | L4 | L1 and 927 | 1 |
| | *DB=EPAB,JPAB,DWPI; PLUR=YES; OP=AND* | | |
| ☐ | L5 | 16172 | 2 |
| ☐ | L6 | 0116172 | 38 |
| ☐ | L7 | moraxella | 410 |
| ☐ | L8 | moraxella.ti. | 136 |
| ☐ | L9 | L8 and 2001 | 71 |

END OF SEARCH HISTORY

The required strains of *E. coli* were cultured overnight with shaking at 37°C in 500mls of luria broth. The cells were pelleted at 5,000 rpm for 15 mins and the supernatant filtered through a 0.45µm filter. Solid ammonium sulfate was added to the supernatant to 60% saturation (180g / 500ml), and dissolved at 4°C with stirring for 30 minutes. This mixture was left at 4°C overnight and the precipitated proteins pelleted at 7,000 rpm for 30 mins. The proteins were resuspended in 3ml of double distilled water and the solubilised proteins dialysed against double distilled water overnight to remove any salt. The resulting mixture was filtered through a 0.45µm filter and stored at -20°C.

Following separation of the proteins by SDS-PAGE, the proteins were transferred to PVDF membrane and excised. The protein was subjected to automated (Edman degradation) sequence analysis (28) with vapour phase delivery of critical reagents (29) in an automated sequenator (model 470A; Applied Biosystems) (Applied Biosystems Division, Foster City, CA, USA) in conjunction with a PTH amino acid separation system (model 120A PTH analyzer; Applied Biosystems).

Using this technique 17 amino acids with two gaps were identified

K E F S Q V I I F G D S L X D X G (SEQ ID NO:7)

which corresponds exactly with amino acids 26 through to 42 shown on the accompanying sequence. This result also indicated that the protein most likely includes an amino terminal signal peptide which is involved in the secretion of the protein. This amino terminal corresponds to amino acids 1 through to 25 in the accompanying sequence.

**Raising antibodies to the lipase in rabbits**

Antibody to the recombinant lipase was raised in rabbits by injecting ammonium sulfate precipitated supernatant from *E. coli* MC1061/pMB4. Prior to vaccination, the lipase preparation was inactivated by heating to 90°C for 90min. 30µg of this protein was injected at 2 weekly intervals for 4 weeks. The primary inoculum was emulsified with Freunds complete adjuvant and subsequent vaccinations with Freunds incomplete adjuvant.

**Heat stability of *M. bovis* lipase**

The recombinant lipase cloned from *M. bovis* Dalton 2d was found to be very heat stable since it required heating at 90°C for 105 minutes for the

| Organism | Protein | Similarity | Identity |
|---|---|---|---|
| *Pasteurella haemolytica* | LktA protein (leukotoxin) | 68% | 50% |
| *Actinobacillus pleuropneumoniae* | RTX toxin determinant | 68% | 48% |
| *Escherichia coli* | Haemolysin - plasmid | 58% | 43% |
| *E. coli* | Haemolysin - chromosomal | 58% | 43% |

**Functional complementation by the *M. bovis* haemolysin**

A construct which expressed the chromosomal-borne haemolysin of *E. coli* was obtained (pLG900; generated by combining the two plasmids pLG575 (26) and pLG816 (*hlyC* and *hlyA* cloned into pBluescriptSK). pLG900 comprises the four genes of the RTX operon, *hlyC, hlyA, hlyB, hlyD,* cloned into pBluescriptSK and is capable of conferring a haemolytic phenotype on *E. coli* cells that were previously non-haemolytic. The A subunit (*hlyA*) of this construct was mutated such that it was no longer able to be expressed but the other genes involved in the operon (*hlyB, hlyC* and *hlyD*) remained intact. The *E. coli* strain containing this construct (pLG900 / *hlyA* negative) was no longer haemolytic. However, the haemolytic phenotype was restored by providing *in trans* the cloned haemolysin subunit gene from *M. bovis* Dalton 2d. Thus it was confirmed that the cloned *M. bovis* haemolysin gene encoded a structural subunit that was most probably a member of the RTX family of haemolytic enzymes.

Further sequence analysis has established that, like other members of the family, the *M. bovis* RTX A subunit gene is flanked by DNA sequences capable of encoding the RTX B,C and D proteins.

**Conservation of the RTX A subunit among *M. bovis***

To determine whether the gene for the RTX A subunit was present in *M. bovis* strains representing the known pilus serotypes, a southern hybridisation analysis was performed using the coding region of the RTX A subunit as a probe.

Genomic DNA extracted from the seven serotype strains of *M. bovis* (15) was digested with *Eco*RV and separated using agarose gel electrophoresis. The DNA was transferred to a Hybond N+ filter (Amersham,

File 155:MEDLINE(R) 1951-2005/Apr W4
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| Set | Items | Description |
|---|---|---|

Cost is in DialUnits
? ds
Terminal set to DLINK
? s rtx (10n) consens?

| Set | Items | Description |
|---|---|---|
| S1 | 549 | 'RTX' |
| S2 | 1511 | GLY (N) GLY |
| S3 | 0 | S1 AND S2 |
| S4 | 0 | S1 AND GGXGXD |
| S5 | 2 | 'GGXGXD' |

? s rtx (10n) consens?

| | | |
|---|---|---|
| | 549 | RTX |
| | 53310 | CONSENS? |
| S6 | 1 | RTX (10N) CONSENS? |

?
? s s6 not s5

| | | |
|---|---|---|
| | 1 | S6 |
| | 2 | S5 |
| S7 | 1 | S6 NOT S5 |

? t s7/9/all

**7/9/1**
DIALOG(R)File 155:MEDLINE(R)
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11430124 PMID: 8757837
**Escherichia coli hemolysin mutants with altered target cell specificity.**
Pellett S; Welch R A
Department of Medical Microbiology and Immunology, University of Wisconsin--Madison 53706, USA.
Infection and immunity (UNITED STATES) Aug 1996, 64 (8) p3081-7, ISSN 0019-9567 Journal Code: 0246127
Contract/Grant No.: AI-20323; AI; NIAID
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
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Subfile: INDEX MEDICUS
In order to understand the functional significance of HlyC-dependent acylation of the Escherichia coli hemolysin structural protein (HlyA), random as well as site-directed substitutions at the known regions of modification, i.e., those at lysine residues at amino acid positions 563 and 689 (HlyAK563 and HlyAK689, respectively), were isolated. Sixteen random hlyA mutations were identified on the basis of a screen for loss of immunoreactivity to the hemolysin-neutralizing D12 monoclonal antibody that reacts to only HlyC-activated HlyA. These substitutions occurred at the region from HlyAE684 to HlyAY696. A recombinant glutathione S-transferase-hemolysin gene fusion encoding glutathione S-transferase-HlyAS608-T725 residues reacts with monoclonal antibody when HlyC is coexpressed with the fusion protein. Therefore, at most only 12% of the total HlyA primary sequence is needed for HlyC-facilitated acylation at the HlyAK689 position, and this modification can occur in the absence of

the proximal HlyAK563 acylation site. The cytolytic activities of these HlyA mutants against sheep erythrocytes and bovine and human lymphocyte cell lines (BL-3 and Raji cells, respectively) were analyzed. HlyAK563 and HlyAK689 substitutions displayed various degrees of loss of cytotoxicity that depended on the particular amino acid replacement. An HlyAK563C variant retained greater than 59 and 21% of its BL-3-lytic and erythrolytic activities, respectively, but was nearly inactive against Raji cells. An HlyA mutant with a K-to-E substitution at amino acid 689 (HlyAK689E) was essentially inactive against all three cell types, whereas an HlyAK689R substitution had a pattern of activity similar to that of the HlyAK563C mutant. Preceding the two in vitro acylated HlyA lysines are glycines that appear to be the only amino acids conserved in alignments of these regions among the RTX toxins. Remarkably, considering the retention of cytotoxic activity by some HlYAK689 mutants, each of three different substitutions at the HlyAG688 position was relatively inactive against all three cell types tested. This suggests that HlyAG688 plays a significant structural role in cytotoxic activity apart from its possible participation in an HlyC activation process which presumably requires recognition of pro-HlyA structures. The related RTX toxin, the Pasteurella haemolytica leukotoxin structural protein (LktA), can be activated in an E. coli recombinant background by HlyC. In amino acid sequence alignments, LktAK554 is equivalent to the HlyAK563 position but it has an asparagine (LktAN684) at the homologous HlyAK689 site. An LktAN684K substitution possesses wild-type leukotoxin activity against BL-3 cells and does not acquire hemolytic or Raji cell cytotoxic activity. Surprisingly, both LktAK554C and LktAK554T substitutions retain considerable BL-3 cytotoxicity (45 and 49%, respectively), indicating that there may be additional lysines within LktA that the HlyC activation mechanism is capable of acylating. Based on these results and a comparison of amino acid sequence alignments of 12 **RTX** toxins, a putative **consensus** structure of the **RTX** residues necessary for HlyC activation is hypothesized.

Tags: Comparative Study; Research Support, U.S. Gov't, P.H.S.

Descriptors: *Acyltransferases; *Bacterial Proteins--toxicity--TO; *Bacterial Toxins--toxicity--TO; *Escherichia coli--pathogenicity--PY; *Escherichia coli Proteins; *Hemolysins--toxicity--TO; *Mutation; *Protein Processing, Post-Translational; Acylation; Amino Acid Sequence; Animals; Bacterial Proteins--genetics--GE; Bacterial Proteins--metabolism--ME; Bacterial Toxins--genetics--GE; Cattle; Dose-Response Relationship, Drug; Escherichia coli--genetics--GE; Exotoxins; Hemolysins--genetics--GE; Hemolysins--metabolism--ME; Hemolysis; Humans; Mannheimia haemolytica --genetics--GE; Mannheimia haemolytica--pathogenicity--PY; Molecular Sequence Data; Phenotype; Recombinant Fusion Proteins--toxicity--TO; Sequence Homology, Amino Acid; Sheep; Structure-Activity Relationship; Toxicity Tests

CAS Registry No.: 0 (Bacterial Proteins); 0 (Bacterial Toxins); 0 (Escherichia coli Proteins); 0 (Exotoxins); 0 (Hemolysins); 0 (Hlya protein, E coli); 0 (Recombinant Fusion Proteins); 0 (leukotoxin)

Enzyme No.: EC 2.3. (Acyltransferases); EC 2.3.1.- (HlyC protein, E coli)

Record Date Created: 19960926

Record Date Completed: 19960926

? logoff hold

27apr05 15:38:18 User228206 Session D2432.3

$1.42 0.443 DialUnits File155

$0.21 1 Type(s) in Format 9

$0.21 1 Types

$1.63 Estimated cost File155

$0.26 TELNET

$1.89 Estimated cost this search

$1.89  Estimated total session cost  0.443 DialUnits

Logoff: level 05.01.00 D  15:38:18

You are now logged off

```
nter an option number to view information or to connect to an online
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(e.g., B1 for ERIC).
? e rtx

       27apr05 15:16:06 User228206 Session D2432.1
            $0.00    0.209 DialUnits FileHomeBase
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File 155:MEDLINE(R) 1951-2005/Apr W4
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E2        1  RTW153Q
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E4        2  RTX 100
E5       17  RTXA
E6        5  RTXA PROTEIN, BACTERIA
E7        1  RTXA PROTEIN, BRADYRHIZOBIUM ELKANII
E8        1  RTXAB
E9        1  RTXAGA
E10       3  RTXB
E11       5  RTXC
E12       2  RTXC PROTEIN, VIBRIO CHOLERAE

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? s e3
      S1     549  'RTX'
? s gly (n) gly
           21696  GLY
           21696  GLY
      S2    1511  GLY (N) GLY
? s s1 and s2
             549  S1
            1511  S2
      S3       0  S1 AND S2
? s s1 and ggxgxd
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               2  GGXGXD
      S4       0  S1 AND GGXGXD
? e ggxgxd

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E2        1  GGXGUAAUAYCC
E3        2 *GGXGXD
E4        2  GGXGXDX
E5        1  GGXGXDXLX
E6        1  GGXP
E7        1  GGXPY
E8        2  GGXXP
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E10         1  GGXYAC
E11         1  GGXYAMIDE
E12        11  GGY

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**5/9/1**

DIALOG(R)File 155:MEDLINE(R)
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13509439 PMID: 10478443

**Identification of a member of the serralysin family isolated from a psychrotrophic bacterium, Pseudomonas fluorescens 114.**

Kumeta H; Hoshino T; Goda T; Okayama T; Shimada T; Ohgiya S; Matsuyama H; Ishizaki K

Graduate School of Science and Engineering, Hokkaido Tokai University, Sapporo, Japan.

Bioscience, biotechnology, and biochemistry (JAPAN) Jul 1999, 63 (7) p1165-70, ISSN 0916-8451 Journal Code: 9205717

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Subfile: INDEX MEDICUS

An extracellular metalloprotease named No. 114 protease is one of the major secretions of a psychrotrophic bacterium, Pseudomonas fluorescens 114, the cold-adaptation mechanism of which has not been identified. In this study, we purified and cloned No. 114 protease, which is a single polypeptide having a molecular mass of 47 kDa. This protease contains a zinc-binding motif (HEXXHXUGUXH: X, arbitrary amino acid; U, bulky hydrophobic amino acid), glycine-rich repeats ( **GGXGXD** ) and no cysteine residue, which are the features specifically found in serralysin subfamily. No. 114 protease has its maximum activity at the temperature of 35-40 degrees C, which is about 20 degrees C lower than that of a serralysin from a mesophilic bacterium, Pseudomonas aeruginosa. All these results imply that No. 114 protease from this psychrophilic bacterium is a unique member of the serralysin group characterized by a low optimal temperature.

Descriptors: *Metalloendopeptidases--chemistry--CH; *Pseudomonas fluorescens--metabolism--ME; Adaptation, Physiological; Amino Acid Sequence ; Base Sequence; Cloning, Molecular; Cold; DNA, Bacterial--biosynthesis--BI ; DNA, Bacterial--genetics--GE; Metalloendopeptidases --isolation and purification--IP; Molecular Sequence Data; Repetitive Sequences, Amino Acid --physiology--PH; Zinc--metabolism--ME

CAS Registry No.: 0 (DNA, Bacterial); 7440-66-6 (Zinc)

Enzyme No.: EC 3.4.24 (Metalloendopeptidases); EC 3.4.24.40 (serralysin)

Record Date Created: 19991102

Record Date Completed: 19991102

**5/9/2**

DIALOG(R)File 155:MEDLINE(R)
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10376916 PMID: 8253063

**Three-dimensional structure of the alkaline protease of Pseudomonas aeruginosa: a two-domain protein with a calcium binding parallel beta roll motif.**

Baumann U; Wu S; Flaherty K M; McKay D B

Beckman Laboratories for Structural Biology, Department of Cell Biology, Stanford University School of Medicine, CA 94305.

EMBO journal (ENGLAND) Sep 1993, 12 (9) p3357-64, ISSN 0261-4189 Journal Code: 8208664

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Subfile: INDEX MEDICUS

The three-dimensional structure of the alkaline protease of Pseudomonas aeruginosa, a zinc metalloprotease, has been solved to a resolution of 1.64 A by multiple isomorphous replacement and non-crystallographic symmetry averaging between different crystal forms. The molecule is elongated with overall dimensions of 90 x 35 x 25 A; it has two distinct structural domains. The N-terminal domain is the proteolytic domain; it has an overall tertiary fold and active site zinc ligation similar to that of astacin, a metalloprotease isolated from a European freshwater crayfish. The C-terminal domain consists of a 21-strand beta sandwich. Within this domain is a novel 'parallel beta roll' structure in which successive beta strands are wound in a right-handed spiral, and in which Ca2+ ions are bound within the turns between strands by a repeated **GGXGXD** sequence motif, a motif that is found in a diverse group of proteins secreted by Gram-negative bacteria.

Tags: Comparative Study; Research Support, Non-U.S. Gov't

Descriptors: *Metalloendopeptidases--chemistry--CH; *Protein Conformation ; *Protein Structure, Secondary; *Pseudomonas aeruginosa--enzymology--EN; Amino Acid Sequence; Animals; Astacoidea; Binding Sites; Calcium --metabolism--ME; Consensus Sequence; Metalloendopeptidases--metabolism--ME ; Models, Molecular; Molecular Sequence Data; Sequence Homology, Amino Acid ; Zinc--metabolism--ME

CAS Registry No.: 7440-66-6 (Zinc); 7440-70-2 (Calcium)

Enzyme No.: EC 3.4.24 (Metalloendopeptidases); EC 3.4.24.21 (astacin)

Record Date Created: 19940110

Record Date Completed: 19940110

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$3.64 Estimated cost this search

$3.64 Estimated total session cost 1.133 DialUnits

Logoff: level 05.01.00 D 15:16:55

You are now logged off

Secreted protease C precursor (EC 3.4.24.-) (ProC). {GENE: Name=prtC} - Erwinia chrysanthemi

RT11_ACTPL (**P55128**)
RTX-I toxin determinant A from serotypes 1/9 (ApxI) (APX-IA) (Hemolysin IA) (HLY-IA) (Cytolysin IA) (CLY-IA). {GENE: Name=apxIA; Synonyms=clyIA, hlyIA} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RT12_ACTPL (**P55129**)
RTX-I toxin determinant A from serotypes 5/10 (APX-IA) (Hemolysin IA) (HLY-IA) (Cytolysin IA) (CLY-IA). {GENE: Name=apxIA; Synonyms=clyIA, hlyIA} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RT1B_ACTPL (**P26760**)
Toxin RTX-I translocation ATP-binding protein (RTX-I toxin determinant B) (APX-IB) (HLY-IB) (Cytolysin IB) (CLY-IB). {GENE: Name=apxIB; Synonyms=appB, clyIB, hlyIB} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RT1C_ACTPL (**P55132**)
RTX-I toxin-activating lysine-acyltransferase apxIC (EC 2.3.1.-) (RTX-I toxin determinant C) (APX-IC) (HLY-IC) (Cytolysin IC) (CLY-IC) (Toxin RTX-I activating protein C). {GENE: Name=apxIC; Synonyms=clyIC, hlyIC} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RT1D_ACTPL (**P26761**)
RTX-I toxin determinant D (APX-ID) (HLY-ID) (Cytolysin ID) (CLY-ID) (Toxin RTX-I secretion protein D). {GENE: Name=apxID; Synonyms=appD, clyID, hlyID} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RT2A_ACTPL (**P15377**)
RTX-II toxin determinant A (APX-IIA) (Hemolysin IIA) (HLY-IIA) (Cytolysin IIA) (CLY-IIA). {GENE: Name=apxIIA; Synonyms=appA, clyIIA, cytC, hlyIIA} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RT2C_ACTPL (**P0A3I3**)
RTX-II toxin-activating lysine-acyltransferase apxIIC (EC 2.3.1.-) (RTX-II toxin determinant C) (APX-IIC) (HLY-IIC) (Cytolysin IIC) (CLY-IIC) (Toxin RTX-II activating protein C). {GENE: Name=apxIIC; Synonyms=appC, ashC, clyIIC, cytC, hlyC} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RT2C_ACTSU (**P0A3I4**)
RTX-II toxin-activating lysine-acyltransferase apxIIC (EC 2.3.1.-) (RTX-II toxin determinant C) (APX-IIC) (HLY-IIC) (Cytolysin IIC) (CLY-IIC) (Toxin RTX-II activating protein C). {GENE: Name=apxIIC; Synonyms=appC, ashC, clyIIC, cytC, hlyC} - Actinobacillus suis

RT31_ACTPL (**P55130**)
RTX-III toxin determinant A from serotype 2 (APX-IIIA) (Cytolysin IIIA) (CLY-IIIA). {GENE: Name=apxIIIA; Synonyms=clyIIIA, ptxA, rtxA} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RT32_ACTPL (**P55131**)
RTX-III toxin determinant A from serotype 8 (APX-IIIA) (Cytolysin IIIA) (CLY-IIIA). {GENE: Name=apxIIIA; Synonyms=clyIIIA, ptxA, rtxA} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RT3B_ACTPL (**Q04473**)
Toxin RTX-III translocation ATP-binding protein (RTX-III toxin determinant B) (APX-IIIB) (Cytolysin IIIB) (CLY-IIIB). {GENE: Name=apxIIIB; Synonyms=clyIIIB, rtxB} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RT3C_ACTPL (**Q04474**)
RTX-III toxin-activating lysine-acyltransferase apxIIC (EC 2.3.1.-) (RTX-III toxin determinant C) (APX-IIIC) (Cytolysin IIIC) (CLY-IIIC) (Toxin RTX-III activating protein C). {GENE: Name=apxIIIC; Synonyms=clyIIIC, rtxC} - Actinobacillus pleuropneumoniae (Haemophilus

pleuropneumoniae)

RT3D_ACTPL (**Q08633**)

RTX-III toxin determinant D (APX-IIID) (Cytolysin IIID) (CLY-IIID) (Toxin RTX-III secretion protein D). {GENE: Name=apxIIID; Synonyms=clyIIID, rtxD} - Actinobacillus pleuropneumoniae (Haemophilus pleuropneumoniae)

RTXC_VIBCH (**Q9X4W3**)

Cytolysin-activating lysine-acyltransferase rtxC (EC 2.3.1.-). {GENE: Name=rtxC; OrderedLocusNames=VC1450} - Vibrio cholerae

[0105] Baumann, U., et al. (1993). Three-dimensional structure of the alkaline protease of Pseudomonas aeruginosa: a two domain protein with a calcium binding parallel beta roll motif. EMBO J. 12:3357-3364

# WEST Search History

Hide Items | Restore | Clear | Cancel

DATE: Wednesday, April 27, 2005

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| ☐ | L7 | GGxGxD | 2 |

END OF SEARCH HISTORY

DNA. 1989 Jan-Feb;8(1):15-28. Related Articles, Links

# DNA sequence of the Pasteurella haemolytica leukotoxin gene cluster.

**Highlander SK, Chidambaram M, Engler MJ, Weinstock GM.**

Department of Biochemistry, University of Texas Medical School, Houston, TX 77030.

Bovine serum was used to identify a recombinant phage clone carrying the Pasteurella haemolytica leukotoxin gene. This fragment produced the 102-kD leukotoxin and several smaller P. haemolytica-specific protein antigens in Escherichia coli. An additional contiguous fragment, containing sequences upstream from the leukotoxin gene. Using these clones, we determined the nucleotide sequence of a 7745-bp region that included four open reading frames: an upstream gene, lktC; the leukotoxin gene, lktA; and two downstream genes, lktB, and lktD. The predicted molecular weights of the proteins encoded by these genes were 19.9, 102, 79.6, and 54.7 kD, respectively. These genes and their predicted proteins were similar in organization and in sequence to the corresponding elements of the gene cluster that encodes an E. coli alpha-hemolysin and its activation and secretion functions. Expression of the leukotoxin was enhanced in E. coli, by fusing the gene to the lac promoter. Under these conditions the leukotoxin was not secreted into the medium, as it is in P. haemolytica. However, in the presence of the alpha-hemolysin genes, the leukotoxin was secreted into the medium, demonstrating functional complementation by the hemolysin secretory system.

PMID: 2707120 [PubMed - indexed for MEDLINE]

L1: Entry 2 of 2 File: USPT Jan 14, 1997

DOCUMENT-IDENTIFIER: US 5594107 A
** See image for Certificate of Correction **
TITLE: Chimeric protein comprising an RTX-family cytotoxin and interferon-2 or interferon

Detailed Description Text (7):
The term "RTX cytotoxin" intends a cytotoxin belonging to the family of cytolytic toxins known as the RTX proteins. The toxins are characterized by a series of repeated amino acid domains near the carboxy terminus. The consensus amino acid sequence is Gly-Gly-X-Gly-(Asn/Asp)-Asp (SEQ ID NO: 5), where X is Lys, Asp, Val or Asn. Such proteins include, among others, leukotoxins derived from Pasteurella and Actinobacillus, such as those found in P. haemolytica, Actinobacillus pleuropneumoniae, A. actinomycetemcomitans, A. suis, as well as the 0 cytotoxins found in Proteus vulgaris, Morganella morganii, Moraxella bovis, Neisseria meningitidis, H. influenzae type B, E. coli alpha hemolysin and Bordetella pertussis adenylate cyclase hemolysin. (For further descriptions of these toxins, see, e.g., Strathdee, C. A., and Lo, R. Y. C. (1987) Infect. Immun. 55: 3233-3236; Lo, R. Y. C. (1990) Can. J. Vet. Res. 54: S33-S35; Welch, R. A. (1991) Mol. Microbiol. 5: 521-528); Lo et al. (1987) Infect. Immun. 55: 1987-1996; Glaser et al. (1988) Molec. Microbiol. 2: 19-30; Lally et al. (1989) J. Biol. Chem. 254: 15451-15456; Kolodrubetz et al. (1989) Infect. Immun. 57: 1465-1469; Chang et al. (1989) DNA 8: 635-647; Frey, J. and Nicolet, J. (1988) Infect. Immun. 56: 2570-2575; Devenish et al. (1989) Infect. Immun. 57: 3210-3213; Koronakis et al. (1987) J. Bacteriol. 169: 1509-1515 and Highlander et al. (1989) DNA 8: 15-28). The desired cytotoxin may be chemically synthesized, isolated from an organism expressing the same, or recombinantly produced.

Detailed Description Text (10):
The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site." One such epitope is the consensus sequence found among the RTX family of toxins described above. This sequence is Gly-Gly-X-Gly-(Asn/Asp)-Asp (SEQ ID NO: 5), where X is preferably Lys, Asp, Val or Asn. Other substitutions for X in the consensus sequence are also contemplated including substitutions with an aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile, a charged amino acid such as Asp, Glu, Arg, His or Lys, or a corresponding neutral amino acid such as Asn or Gln.

Detailed Description Text (126):
As explained above, the P. haemolytica leukotoxin protein is a member of the RTX family of toxins and contains a series of repeated amino acid domains near the carboxy terminus. These domains are likely to be epitopes useful in the subject chimeric proteins. The consensus amino acid sequence is Gly-Gly-X-Gly-(Asn or Asp)-Asp (SEQ ID NO: 5), where X is Lys, Asp, Val or Asn. (Highlander et al. (1989) DNA 8: 15-28; Welch, R. A. (1991) Molec. Microbiol. 5: 521-528). However, other substitutions likely to render immunologically active peptides include substitutions with an aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile, a charged amino acid such as Asp, Glu, Arg, His or Lys, or a corresponding neutral amino acid such as Asn or Gln.

CLAIMS:

1. An immunogenic chimeric protein comprising a cytokine selected from the group consisting of interleukin-2 (IL2), and gamma-interferon (.gamma.IFN), linked to at least one epitope of an RTX cytotoxin which comprises the amino acid sequence Gly-Gly-X-Gly-(Asn or Asp)-Asp (SEQ ID NO: 5), wherein X is selected from the group consisting of an aliphatic amino acid, and a charged amino acid or

its corresponding neutral amino acid.

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L1: Entry 1 of 2 File: USPT Aug 1, 2000

DOCUMENT-IDENTIFIER: US 6096320 A
TITLE: Vaccines with chimeric protein comprising gamma-interferon and leukotoxin derived from pasteurella haemolytica

Detailed Description Text (8):
The term "RTX cytotoxin" intends a cytotoxin belonging to the family of cytolytic toxins known as the RTX proteins. The toxins are characterized by a series of repeated amino acid domains near the carboxy terminus. The consensus amino acid sequence is Gly-Gly-X-Gly-(Asn/Asp)-Asp (SEQ ID NO:5), where X is Lys, Asp, Val or Asn. Such proteins include, among others, leukotoxins derived from Pasteurella and Actinobacillus, such as those found in P. haemolytica, Actinobacillus pleuropneumoniae, A. actinomycetemcomitans, A. suis, as well as the cytotoxins found in Proteus vulgaris, Morganella morganii, Moraxella bovis, Neisseria meningitidis, H. influenzae type B, E. coli alpha hemolysin and Bordetella pertussis adenylate cyclase hemolysin. (For further descriptions of these toxins, see, e.g., Strathdee, C. A., and Lo, R. Y. C. (1987) Infect. Immun. 55:3233-3236; Lo, R. Y. C. (1990) Can. J. Vet. Res. 54:S33-S35; Welch, R. A. (1991) Mol. Microbiol. 5:521-528); Lo et al. (1987) Infect. Immun. 55:1987-1996; Glaser et al. (1988) Molec. Microbiol. 2:19-30; Lally et al. (1989) J. Biol. Chem. 254:15451-15456; Kolodrubetz et al. (1989) Infect. Immun. 57:1465-1469; Chang et al. (1989) DNA 8:635-647; Frey, J. and Nicolet, J. (1988) Infect. Immun. 56:2570-2575; Devenish et al. (1989) Infect. Immun. 57:3210-3213; Koronakis et al. (1987) J. Bacteriol. 169:1509-1515 and Highlander et al. (1989) DNA 8:15-28). The desired cytotoxin may be chemically synthesized, isolated from an organism expressing the same, or recombinantly produced.

Detailed Description Text (11):
The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site." One such epitope is the consensus sequence found among the RTX family of toxins described above. This sequence is Gly-Gly-X-Gly-(Asn/Asp)-Asp (SEQ ID NO:5), where X is preferably Lys, Asp, Val or Asn. Other substitutions for X in the consensus sequence are also contemplated including substitutions with an aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile, a charged amino acid such as Asp, Glu, Arg, His or Lys, or a corresponding neutral amino acid such as Asn or Gln.

Detailed Description Text (131):
As explained above, the P. haemolytica leukotoxin protein is a member of the RTX family of toxins and contains a series of repeated amino acid domains near the carboxy terminus. These domains are likely to be epitopes useful in the subject chimeric proteins. The consensus amino acid sequence is Gly-Gly-X-Gly-(Asn or Asp)-Asp, (SEQ ID NO:5) where X is Lys, Asp, Val or Asn. (Highlander et al. (1989) DNA 8:15-28; Welch, R. A. (1991) Molec. Microbiol. 5:521-528). However, other substitutions likely to render immunologically active peptides include substitutions with an aliphatic amino acid, such as Gly, Ala, Val, Leu, Ile, a charged amino acid such as Asp, Glu, Arg, His or Lys, or a corresponding neutral amino acid such as Asn or Gln.

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L5: Entry 24 of 24 File: USPT Jun 6, 1995

DOCUMENT-IDENTIFIER: US 5422110 A
TITLE: Enhanced immunogenicity using leukotoxin chimeras

Detailed Description Text (7):
The term "leukotoxin polypeptide" intends a polypeptide derived from a protein belonging to the family of molecules characterized by the carboxy-terminus consensus amino acid sequence Gly-Gly-X-Gly-X-Asp (SEQ ID NO:11) (Highlander et al., DNA (1989) 8:15-28), where X is Lys, Asp, Val or Asn. Such proteins include, among others, leukotoxins derived from P. haemolytica and Actinobacillus pleuropneumoniae, as well as E. coli alpha hemolysin (Strathdee, C. A., and Lo, R. Y. C. Infect. Immun. (1987) 55:3233-3236; Lo, R. Y. C., Can. J. Vet. Res. (1990) 54:S33-S35; Welch, R. A., Mol. Microbiol. (1991) 5:521-528). This family of toxins is known as the "RTX" family of toxins (Lo, R. Y. C., Can. J. Vet. Res. (1990) 54:S33-S35). In addition, the term "leukotoxin polypeptide" refers to a leukotoxin polypeptide which is chemically synthesized, isolated from an organism expressing the same, or recombinantly produced. Furthermore, the term intends an immunogenic protein having an amino acid sequence substantially homologous to a contiguous amino acid sequence found in the particular native leukotoxin molecule. Thus, the term includes both full-length and partial sequences, as well as analogs. Although native full-length leukotoxins display leukotoxic activity, the term "leukotoxin" also intends molecules which remain immunogenic yet lack the cytotoxic character of native leukotoxins. The nucleotide sequences and corresponding amino acid sequences for several leukotoxins are known. See, e.g., U.S. Pat. Nos. 4,957,739 and 5,055,400; Lo et al., Infect. Immun. (1985) 50:667-67; Lo et al., Infect. Immun. (1987) 55:1987-1996; Strathdee, C. A., and Lo, R. Y. C., Infect. Immun. (1987) 55:3233-3236; Highlander et al., DNA (1989) 8:15-28; Welch, R. A., Mol. Microbiol. (1991) 5:521-528.

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08058098 PMID: 3683855

**Glycine immunoreactivity localized in the cochlear nucleus and superior olivary complex.**

Wenthold R J; Huie D; Altschuler R A; Reeks K A

Laboratory of Neuro-otolaryngology, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD 20892.

Neuroscience (ENGLAND) Sep 1987, 22 (3) p897-912, ISSN 0306-4522 Journal Code: 7605074

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Subfile: INDEX MEDICUS

Polyclonal antibodies were made in rabbits against glycine conjugated to bovine serum albumin with glutaraldehyde and were used for immunocytochemical studies in the cochlear nucleus and superior olivary nucleus of the guinea-pig. Antibodies selective for glycine were prepared by affinity chromatography. By dot-blot analysis this preparation showed a strong recognition of glycine conjugates and relatively little recognition of conjugates of most other amino acids tested. However, there was a significant reaction with conjugates of alanine and beta-alanine, and this cross-reaction could not be removed by affinity chromatography without eliminating the preparation's recognition of glycine. The affinity-purified preparation showed only a weak recognition of conjugates of gamma-aminobutyrate (GABA) which was detectable at high concentrations of primary antibody. Immunocytochemical studies showed several intensely staining cell bodies in the cochlear nucleus and superior olivary complex. Most immunoreactive cell bodies in the cochlear nucleus were in the dorsal cochlear nucleus, being present in both the superficial and deep layers. Scattered immunoreactive cells were present in the ventral cochlear nucleus. Intense staining of cell bodies was seen in the medial nucleus of the trapezoid body, and these cells appear to correspond to the principal cells of that nucleus. Punctate labelling, suggestive of immunoreactive presynaptic terminals, was also apparent, particularly in the ventral cochlear nucleus and lateral superior olive. In the ventral cochlear nucleus, immunoreactive puncta were found around unlabeled cell bodies, at times nearly covering the perimeter of the cell. A population of glycine-immunoreactive cell bodies in the superficial dorsal cochlear nucleus also labeled with anti-GABA antibodies as determined through double-labeling studies. However, glycine-positive cells in the deep dorsal cochlear nucleus were not labeled with anti-GABA antibodies, and some populations of GABA-positive cells in the superficial layers were not labeled with **anti - glycine antibodies** . In the hippocampus intense staining of cell bodies and puncta was seen with anti-GABA antibodies while essentially no staining was seen with **anti - glycine antibodies** . These results suggest that **anti - glycine antibodies** can be useful for immunocytochemical identification of glycinergic neurons. From this study several populations of putative glycinergic neurons are identified in the auditory nuclei of the brain stem using these antibodies. Some populations of GABA-containing neurons also contain high levels of glycine or a related molecule.

Tags: Female

INFECTION AND IMMUNITY, Aug. 1996, p. 3081–3087
0019-9567/96/$04.00+0
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Vol. 64, No. 8

# *Escherichia coli* Hemolysin Mutants with Altered Target Cell Specificity

SHAHAIREEN PELLETT AND RODNEY A. WELCH*

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**In order to understand the functional significance of HlyC-dependent acylation of the *Escherichia coli* hemolysin structural protein (HlyA), random as well as site-directed substitutions at the known regions of modification, i.e., those at lysine residues at amino acid positions 563 and 689 ($HlyA_{K563}$ and $HlyA_{K689}$, respectively), were isolated. Sixteen random *hlyA* mutations were identified on the basis of a screen for loss of immunoreactivity to the hemolysin-neutralizing D12 monoclonal antibody that reacts to only HlyC-activated HlyA. These substitutions occurred at the region from $HlyA_{E684}$ to $HlyA_{Y696}$. A recombinant glutathione *S*-transferase–hemolysin gene fusion encoding glutathione *S*-transferase–$HlyA_{S608\text{-}T725}$ residues reacts with monoclonal antibody when HlyC is coexpressed with the fusion protein. Therefore, at most only 12% of the total HlyA primary sequence is needed for HlyC-facilitated acylation at the $HlyA_{K689}$ position, and this modification can occur in the absence of the proximal $HlyA_{K563}$ acylation site. The cytolytic activities of these HlyA mutants against sheep erythrocytes and bovine and human lymphocyte cell lines (BL-3 and Raji cells, respectively) were analyzed. $HlyA_{K563}$ and $HlyA_{K689}$ substitutions displayed various degrees of loss of cytotoxicity that depended on the particular amino acid replacement. An $HlyA_{K563C}$ variant retained greater than 59 and 21% of its BL-3-lytic and erythrolytic activities, respectively, but was nearly inactive against Raji cells. An HlyA mutant with a K-to-E substitution at amino acid 689 ($HlyA_{K689E}$) was essentially inactive against all three cell types, whereas an $HlyA_{K689R}$ substitution had a pattern of activity similar to that of the $HlyA_{K563C}$ mutant. Preceding the two in vitro acylated HlyA lysines are glycines that appear to be the only amino acids conserved in alignments of these regions among the RTX toxins. Remarkably, considering the retention of cytotoxic activity by some $HlyA_{K689}$ mutants, each of three different substitutions at the $HlyA_{G688}$ position was relatively inactive against all three cell types tested. This suggests that $HlyA_{G688}$ plays a significant structural role in cytotoxic activity apart from its possible participation in an HlyC activation process which presumably requires recognition of pro-HlyA structures. The related RTX toxin, the *Pasteurella haemolytica* leukotoxin structural protein (LktA), can be activated in an *E. coli* recombinant background by HlyC. In amino acid sequence alignments, $LktA_{K554}$ is equivalent to the $HlyA_{K563}$ position but it has an asparagine ($LktA_{N684}$) at the homologous $HlyA_{K689}$ site. An $LktA_{N684K}$ substitution possesses wild-type leukotoxin activity against BL-3 cells and does not acquire hemolytic or Raji cell cytotoxic activity. Surprisingly, both $LktA_{K554C}$ and $LktA_{K554T}$ substitutions retain considerable BL-3 cytotoxicity (45 and 49%, respectively), indicating that there may be additional lysines within LktA that the HlyC activation mechanism is capable of acylating. Based on these results and a comparison of amino acid sequence alignments of 12 RTX toxins, a putative consensus structure of the RTX residues necessary for HlyC activation is hypothesized.**

The *Escherichia coli* hemolysin is an exotoxin capable of inducing metabolically disruptive $Ca^{2+}$ influxes and eventual lysis of a variety of cell types from different hosts (for a review, see reference 31). Among the different pathogenic types of *E. coli*, this toxin is produced by the most common isolates that cause upper urinary tract infections in both men and women without underlying disease or anatomical complications (for a review, see reference 6). The *E. coli* hemolysin is a member of the RTX family of cytotoxins which are produced by a variety of gram-negative animal and human pathogens, including members of the following genera: *Pasteurella*, *Actinobacillus*, *Bordetella*, *Proteus*, *Morganella*, and possibly *Moraxella* (31).

Controversy exists over the presence of lipopolysaccharide in the large, >300-kDa lytic *E. coli* hemolysin complex (5, 29). It is clear, however, that the only polypeptide component is the 110-kDa product of the *hlyA* gene. In order to be cytotoxic, the HlyA protein must be modified through the poorly understood activity of a second, cotranscribed gene product, HlyC. The chemical nature of the modification was recently discovered to be an amide-linked acylation (27). The sites of acylation for in vitro modified HlyA of murine *E. coli* origin occur at lysine residues at amino acid positions 564 and 690 in HlyA ($HlyA_{K564}$ and $HlyA_{K690}$, respectively) (27). It is unknown if these sites are uniformly modified in vivo. The adenylate cyclase/hemolysin of *Bordetella pertussis* shares sequence similarity to the *E. coli* hemolysin and is also acylated by an HlyC homolog (2). The in vivo adenylate cyclase/hemolysin is acylated at only a single lysine residue ($CyaA_{K983}$), which by sequence alignments corresponds to $HlyA_{K690}$ (11). When the adenylate cyclase/hemolysin is expressed in a recombinant form in an *E. coli* background, the $CyaA_{K680}$ position (equivalent to the $HlyA_{K564}$ position) is acylated approximately 66% of the time (12). Interestingly, this form of the adenylate cyclase/hemolysin is less hemolytic than the wild-type toxin but maintains its natural cell-invasive, adenylate cyclase-mediated levels of toxicity. This suggests that in some instances, acylation

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TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Source or reference |
|---|---|---|
| DH1 | Background strain | 17 |
| C600 | Background strain | 1 |
| JM101 | Background strain | 19 |
| CJ236 | *dut-1 ung-1 thi-1 relA1* | 14 |
| pSF4000 | *hlyCABD* from J96 in pACYC184 | 32 |
| pWAM04 | *hlyCABD* subcloned from pSF4000 in pUC19 | 33 |
| pWAM1100 | *hlyA* from J96 in pBS+ | 25 |
| pWAM1111 | *lktA* from pWAM828[a] in pBS+ | This study |
| pWAM974 | *hlyCBD* from J96 in pACYC184 | 25 |
| pANN202-812 | *hlyCABD* from pHLY152 in pBR322 | 18 |
| pANN202-812AL5* | $HlyA_{\Delta 238\text{-}259}$ | 18 |
| pANN202-812FG10* | $HlyA_{K690M}$ | 18 |
| pANN202-312AL10-1* | $HlyA_{\Delta repeat\ 5}$ | 18 |
| pGEX-2T | GST fusion vector | 26 |

[a] From Forestier and Welch (9).

of specific lysines of an RTX toxin may adversely affect cytotoxic activities.

In this study we examined the cytotoxic phenotypes of *E. coli* hemolysin mutants possessing substitutions at and around the known in vitro sites of HlyA lysine acylation. In addition, we examined the effects of substitutions at the equivalent sites in the *Pasteurella haemolytica* leukotoxin, which has significant amino acid sequence similarity with the *E. coli* hemolysin but which, unlike the broad range of susceptible target cells for the hemolysin, is toxic to only leukocytes of ruminant origin. These studies revealed that hemolysin target cell specificity can be influenced by amino acid substitutions at the acylation sites and that it is likely that the leukotoxin is acylated at a site besides those equivalent to the $HlyA_{K564}$ and $HlyA_{K680}$ positions.

## MATERIALS AND METHODS

**Strains and media.** Bacterial strains and recombinant plasmids and vectors used in this study are summarized in Table 1.

Unless noted otherwise, chemicals were acquired from Sigma (St. Louis, Mo.) and formulations of media used in this laboratory were described previously (21, 25). LB and YT media were used and supplemented with appropriate antibiotics (ampicillin [100 μg/ml] and chloramphenicol [20 μg/ml]) as previously described (9).

**Mutagenesis.** Site-directed mutagenesis was performed essentially by the U-template method as described before, with either *hlyA* or *lktA* recombinant subclones in M13 phage or pBS+ phagemid vectors (9, 25). Screening of potential mutated DNAs was performed by oligonucleotide hybridization with a commercially purchased enhanced chemiluminescence 3′ oligonucleotide-labeling and detection system (ECL system; Amersham, Chicago, Ill.).

Random mutagenesis was performed by two different methods. $NH_2OH$ mutagenesis was performed as described by Miller (20). Dye-buoyant density ultracentrifugation-purified pWAM1100 DNA (*hlyA* in pBS+) (25) was mixed with a solution containing 100 μl of 0.5 M $KPO_4$–5 mM EDTA, (pH 6.0), 200 μl of 1 M $NH_2OH$, and 200 μl of double-distilled $H_2O$ and was incubated at 37°C for periods of time varying from 16 to 36 h. The treated DNA was then dialyzed against 2 liters of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA (TE buffer) for 4 h at 4°C. The DNA was ethanol precipitated and resuspended in TE buffer in a volume sufficient to concentrate the DNA approximately 50-fold. The DNA was then used to transform WAM974 (DH1 with a pACYC184-based recombinant encoding *hlyCBD* [25]), and the resulting transformants were screened for altered beta-hemolytic phenotypes on sheep erythrocyte agar plates containing ampicillin and chloramphenicol. Representatives of all observed phenotypes from those with no zones of hemolysis to those with hemolysis zones two to three times greater in diameter than the wild-type zones were patched to LB agar plates containing chloramphenicol and ampicillin. The resulting cell growth after overnight incubation at 37°C was used for immunoprobing by a colony lift procedure modified from the method described in the procedures manual for the Amersham ECL detection system. Three different antibody preparations were used in this analysis. To ensure that the HlyA antigen was still being produced by potential mutant strains, rabbit anti-HlyA serum was used and only candidates with an unaltered immunobased signal to this polyclonal reagent were chosen for further study. To detect alterations in the epitopes associated with the $HlyA_{K689}$ acylation site and the HlyA $Ca^{2+}$ binding domain, the murine monoclonal antibodies (MAbs) D12 and A10, respectively, were used (23, 25). Of special note is that the blocking buffer for nitrocellulose filters probed with either the polyclonal or A10 antibodies was phosphate-buffered saline (PBS) plus 0.1% Tween 20. However, filters probed with the MAb D12 were probed with PBS plus 5% powdered skim milk because the PBS-Tween blocking buffer prevents detection of D12 reactivity with the HlyC-activated form of the HlyA antigen. Development and detection of antibody reactivity were performed as described in the ECL manual. Controls for loss of reactivity of the D12 and A10 MAbs were provided by including cells from WAM783 (HlyABD) and WAM713 ($HlyCA_{\Delta D728\text{-}A829}BD$ [HlyCABD with the region D-728 to A-829 deleted from the A locus]), respectively (23). Candidate HlyA mutant proteins that were still reactive to anti-HlyA polyclonal serum but which were nonreactive to D12 and/or A10 MAbs were then subjected to immunoblotting (23) in order to examine the relative size and quantity of the mutant HlyA forms expressed. The locations of *hlyA* mutations in the candidate mutants were initially determined by isolating and purifying the *hlyA*-containing plasmid DNA and isolating a 703-bp *Bpu*1102I-*Bgl*II restriction endonuclease fragment which was then substituted for the same fragment from the original pWAM1100 in a ligation reaction. The ligated DNA was then used to transform WAM974, and transformants were reexamined for their hemolytic and antibody phenotypes. The mutations associated with the *Bpu*1102I-*Bgl*II fragment were identified by DNA sequence analysis as previously described (25).

The second random mutagenesis method, based on misincorporation of bases during PCR DNA amplification, was used with modifications (15). Purified pWAM1100 DNA was used as a template for amplification with one oligomer corresponding to bp 3022 to 3042 being combined with either of a pair of oligomers corresponding to the reverse complement of bp 3582 to 3599 or 3876 to 3890 (8). To increase the chances of misincorporation of bases, the concentration of individual deoxynucleoside triphosphates (dNTPs) in the reactions was reduced from the typical 250 μM normally used to a variety of concentrations, down to as low as 37 μM dNTPs. The amplified DNA fragments were then digested with *Bpu*1102I and either *Mlu*I with the use of the oligomer from bp 3599 to 3582 or *Bgl*II in the case of the oligomer from bp 3876 to 3890. The resultant fragments were then substituted in pWAM1100, and mutation analysis was performed as described above.

**Hemolysis.** The lytic capability of wild-type hemolysin and the different HlyA mutant proteins against sheep erythrocytes (hemolysis) was assessed by the recently elaborated method of Bauer and Welch (3). Briefly, this involves twofold serial dilution of toxin-containing culture supernatants, spectrophotometric measurement of the hemoglobin released after a 1-h incubation of the toxin dilutions with a 1% suspension of sheep erythrocytes, construction of a dose-response graph, and calculation of the slope of hemolytic activity using a line drawn from the first three toxin doses that gave hemolysis levels above those of the background controls.

**Cytotoxicity.** Two different cultured cell lines, BL-3 (bovine lymphoma) and Raji (human B-cell lymphoma), were used in $^{51}Cr$ release assays of toxin cellular lysis. The growth, maintenance, and radiolabeling of these cells were performed as previously described (3). Toxin-containing filtered culture supernatants were serially diluted twofold in HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid)-buffered saline (0.8% NaCl–0.04% KCl–0.1% sucrose–10 mM HEPES [pH 7.4]–6 mM $CaCl_2$) (HBS-$CaCl_2$), approximately $2 \times 10^5$ $^{51}Cr$-labeled target cells were added to each toxin dilution, and the mixtures were incubated for 1 h at 37°C. The unlysed cells were removed by centrifugation at $740 \times g$, and the amount of lysis was determined by measuring the released $^{51}Cr$ in terms of counts per minute. To determine total cell lysis, cells were lysed with 0.7 M HCl, and background release of $^{51}Cr$ was determined from incubation of the cells with just HBS-$CaCl_2$. The following formula was used to calculate percent lysis: 100 × (sample cpm − background cpm)/(total cpm − background cpm), where cpm is $^{51}Cr$ release in counts per minute.

**Electrophoresis and immunoblotting.** The trichloroacetic acid precipitation of HlyA and LktA proteins from toxin-containing culture supernatants and sample preparation for sodium dodecyl sulfate–10% polyacrylamide electrophoresis (SDS-PAGE) were performed as described previously (3). Proteins separated by electrophoresis were prepared for immunoblotting essentially by the method of Towbin et al. (28). Nitrocellulose blots were blocked and probed with either rabbit polyclonal antibodies or murine MAbs, and antigen-antibody complexes were detected as described recently (3).

HlyA and LktA in culture supernatants were quantified as follows. Culture supernatant samples were concentrated by trichloroacetic acid precipitation, the samples were serially diluted twofold, each dilution was subjected to SDS-PAGE, and the separated proteins were stained with Coomassie brilliant blue. The intensity of the stained polypeptides present in dried gels was measured by a scanning densitometer and compared with that of serial dilutions of a known amount of commercially purified β-galactosidase run on the same gel.

## RESULTS

The structural and functional significance of the HlyC-facilitated acylation of the *E. coli* hemolysin is poorly understood. The discovery by Stanley et al. (27) of in vitro acylation of $HlyA_{K564}$ and $HlyA_{K690}$ residues by an apparently novel mechanism combined with the localization of the HlyC-dependent anti-HlyA D12 MAb HlyA epitope between $HlyA_{V673}$ and $HlyA_{R726}$ ($HlyA_{V673\text{-}R726}$) (25) prompted our studies to better define the D12 epitope and the functional consequences of substitutions at and around the $HlyA_{K564}$ and $HlyA_{K690}$ positions. It is instructive to indicate that our numbering of HlyA residues differs from that of Stanley et al. because the *E. coli* sources of the *hlyA* genes differ and these genes are not entirely identical (8, 13). Consequently, the HlyA numbering for the sequence studied in Great Britain has the acylated lysines at positions 564 and 690 whereas the homologous positions reported in this work are numbered 563 and 689. The results of the mutagenesis and phenotypic studies are summarized in Tables 2 and 3. Hydroxylamine mutagenesis of the entire *hlyA* gene resulted in identification of six independent substitutions ($HlyA_{E684K}$, $HlyA_{G688R}$ [occurred twice], $HlyA_{G688E}$ [occurred twice], and $HlyA_{G688K}$) in which there is loss of the MAb D12 anti-HlyA reactivity without loss of the neighboring, distal A10 MAb epitope (23, 25). These results led to the isolation of additional HlyA substitutions by focusing mutagenesis efforts on just the *hlyA* sequences encoding the $HlyA_{S391\text{-}A829}$ region. PCR-based mutagenesis resulted in 10 independent mutants lacking the HlyA D12 epitope. Of 10 PCR-generated *hlyA* mutations, 4 represented single amino acid substitutions ($HlyA_{K689R}$, $HlyA_{K689E}$, and $HlyA_{T694P}$ [occurred twice]). The remaining 6 mutations represented double HlyA substitutions in which one of the substitutions fell within the $HlyA_{S686\text{-}Y696}$ span and the other substitution was present 11 to 28 positions away (e.g., $HlyA_{Y696C,T724A}$ and $HlyA_{T666A,G688R}$). Thus, the location of HlyA substitutions that affected loss of the HlyC-dependent D12 MAb reactivity coincided with the 12-amino-acid $HlyA_{E684\text{-}Y696}$ region within which $HlyA_{K689}$ represents one of the two lysines acylated in the in vitro system described by Stanley et al. (27). This is also the homologous site for in vivo acylation of the *B. pertussis* adenylate cyclase/hemolysin (11). Oligonucleotide site-directed mutagenesis was employed to isolate an $HlyA_{K689C}$ substitution for this study and for use in thiol-derivatization experiments that will be reported elsewhere (22). This HlyA mutant protein is also no longer reactive to the D12 MAb. Lastly, the $HlyA_{K690M}$ substitution isolated by Ludwig et al. (18) was found to be nonreactive to the D12 MAb in contrast to the wild-type HlyA encoded by the recombinant plasmid pANN202-312.

To test the hypothesis that the apparent acylation at the proximal $HlyA_{K563}$ site is required for creation of the HlyC-dependent D12 epitope at the distal $HlyA_{K689}$ position, two different $HlyA_{K563}$ substitutions were isolated by oligonucleotide site-directed mutagenesis. Neither the $HlyA_{K563T}$ nor the $HlyA_{K563C}$ substitutions are affected in their D12 reactivity. This observation supports the earlier report that at least in *cis* configuration, the $HlyA_{K689}$ modification associated with the D12 epitope does not require any portion of the $HlyA_{M1\text{-}A626}$ sequence (25). We were also curious as to the HlyA structural context required for the HlyC-dependent HlyA modification and expression of reactivity to the D12 MAb. In other words, could a small portion of the HlyA polypeptide containing the $HlyA_{K689}$ region be recognized in vivo by the HlyC activation mechanism? To initiate these studies, glutathione *S*-transferase–hemolysin gene fusions were constructed in back-

TABLE 2. Summary of *hlyA* and *lktA* mutations in this study

| pWAM no. and mutant | WAM no.[a] | bp change[b] | Substitution | Mutagenesis method |
|---|---|---|---|---|
| *hlyA* | | | | |
| 2158 | 2160 | 3005 A→T<br>3007 A→T<br>3008 A→C | K563T | SDM[c] |
| 2119 | 2121 | 3006 A→T<br>3007 A→G<br>3008 A→C | K563C | SDM |
| 2229 | 1819 | 3371 G→A | E684K | $NH_2OH$[d] |
| 1911 | 1918 | 3381 G→A[e] | G688R | $NH_2OH$ |
| 1912 | 1919 | 3382 G→A[e] | G688E | $NH_2OH$ |
| 1913 | 1920 | 3381 G→A<br>3382 G→A | G688K | $NH_2OH$ |
| 1914 | 1921 | 3385 A→G | K689R | PCR[f] |
| 1915 | 1922 | 3384 A→G | K689E | PCR |
| 2120 | 2122 | 3384 A→T<br>3385 A→G<br>3386 A→C | K689C | SDM |
| 1916 | 1923 | 3399 A→C | T694P | PCR |
| 1917 | 1924 | 3399 A→C<br>3683 A→G | T694P | PCR |
| 2149 | 2156 | | K563C<br>K689C | Cloning[g] |
| 2108 | 2106 | 3302 T→C<br>3307 A→G<br>3320 T→A<br>3399 A→C | Y663C<br>T694P | PCR |
| 2233 | 2237 | 3315 A→G<br>3381 G→A | T666A<br>G688R | PCR |
| 2232 | 2236 | 3319 G→A<br>3375 T→C | R667H<br>S686P | PCR |
| 2231 | 2235 | 3381 G→A<br>3403 A→G | G688R<br>Q695R | PCR |
| 2107 | 2105 | 3406 A→G<br>3489 T→C | Y696C<br>T724A | PCR |
| 2230 | 2234 | 3404 A→G<br>3405 T→C<br>3451 A→G | Y696H<br>E711G | PCR |
| *lktA* | | | | |
| 2181 | 2182 | 2804 A→T<br>2805 A→G<br>2806 A→C | K554C | SDM |
| 2157 | 2159 | 2805 A→C<br>2806 A→C | K554T | SDM[h] |
| 2187 | 2188 | 3196 C→G | N684K | |
| 2183 | 2184 | | K554C<br>N684K | Cloning[i] |

[a] Strain designation of *hlyA* in *trans* to *hlyCBD*.
[b] *hlyA* base pair numbering from reference 8; *lktA* base pair numbering from reference 16.
[c] SDM, site-directed mutagenesis as described in the text.
[d] $NH_2OH$ refers to random mutagenesis by hydroxylamine as described in the text.
[e] This mutation was isolated twice independently.
[f] PCR, PCR random mutagenesis as described in the text.
[g] Restriction endonuclease fragments containing pWAM2119 and pWAM2120 mutations to make the double mutation.
[h] Site-directed mutagenesis mutation originally described in reference 9.
[i] Mutagenesis as described in footnote *f* above but using plasmids pWAM2181 and pWAM2187.

TABLE 3. Lysis of sheep erythrocytes and BL-3 and Raji cells by different *E. coli* hemolysin and *P. haemolytica* leukotoxin mutants

| HlyA or LktA form | % Lysis (mean ± SEM) | | | D12 MAb reactivity[b] |
|---|---|---|---|---|
| | sRBC[a] | BL-3 cell | Raji cell | |
| HlyA | 90 ± 5 | 100 ± 0 | 60 ± 9 | + |
| pro-HlyA | 0 | 0 | 0 | − |
| $HlyA_{K563T}$ | 0 | 45 ± 11 | 0 | + |
| $HlyA_{K563C}$ | 21 ± 1 | 59 ± 11 | 2 ± 1 | + |
| $HlyA_{E684K}$ | 92 ± 5 | 89 ± 9 | 53 ± 9 | −[c] |
| $HlyA_{G688R}$ | 5 ± 2 | 11 ± 8 | 2 ± 1 | − |
| $HlyA_{G688E}$ | 3 ± 1 | 14 ± 7 | 3 ± 3 | − |
| $HlyA_{G688K}$ | 4 ± 2 | 11 ± 6 | 0 | − |
| $HlyA_{K689R}$ | 11 ± 2 | 41 ± 13 | 0 | − |
| $HlyA_{K689E}$ | 0 | 2 ± 1 | 2 ± 2 | − |
| $HlyA_{K689C}$ | 0 | 2 ± 1 | 3 ± 3 | − |
| $HlyA_{K563C,K689C}$ | 0 | 1 ± 1 | 0 | NT |
| $HlyA_{T694P}$ | 0 | 44 ± 7 | 0 | − |
| $HlyA_{Y696F}$ | 86 ± 2 | 86 ± 6 | 28 ± 4 | +[c] |
| $HlyA_{\Delta 626-673}$ | 0 | 0 | 0 | +[c] |
| $HlyA_{\Delta 673-699}$ | 0 | 0 | 0 | −[c] |
| HlyA*[d] | 85 ± 5 | 99 ± 0 | 54 ± 9 | + |
| $HlyA^*_{K690M}$ | 0 | 41 ± 8 | 2 ± 1 | − |
| $HlyA^*_{\Delta 238-259}$ | 0 | 2 ± 1 | 0 | NT |
| $HlyA^*_{\Delta repeat\ \#5}$ | 89 ± 4 | 60 ± 2 | 14 ± 6 | NT |
| LktA | 0 | 76 ± 4 | 0 | − |
| $LktA_{K554T}$ | 0 | 49 ± 13 | 0 | NT |
| $LktA_{K554C}$ | 0 | 45 ± 5 | 0 | NT |
| $LktA_{N684K}$ | 0 | 79 ± 3 | 0 | − |
| $LktA_{K554C,N684K}$ | 0 | 47 ± 4 | 0 | NT |

[a] sRBC, sheep erythrocyte.
[b] +, positive for reactivity; −, negative for reactivity; NT, not tested.
[c] D12 reactivity reported previously (25); lytic assay reperformed for the purpose of side-by-side comparison.
[d] HlyA* is the allelic form of HlyA expressed from recombinant plasmids encoding the *hly* genes from pHly152 (18).

grounds with or without *hlyC* recombinant plasmids in *trans*. Cellular lysates from a fusion containing $HlyA_{S608-T725}$ residues were tested for reactivity to anti-HlyA polyclonal antibodies and MAbs. Shown in Fig. 1 are immunoblots of these fusions demonstrating the HlyC dependence for the D12 epitope by HlyC-independent reactivity to a second anti-HlyA MAb, D1, which has an epitope proximal to that of D12 in the $HlyA_{S608-T725}$ sequence (25).

Stanley et al. reported that arginine and leucine substitutions at either of the two acylated lysines result in loss of in vivo hemolytic activity in culture supernatants (27). The different HlyA substitution mutants isolated in this study were initially scored for their hemolytic phenotype by growth on sheep erythrocyte agar plates. We observed that with the *hlyA* mutations studied in this laboratory, a mutant with an $HlyA_{K689R}$ substitution homologous to that described above loses its D12 reactivity but retains a small discernible zone of beta-hemolysis surrounding colonies grown on blood plates. To examine this observation further, the effects of the new *hlyA* mutations on cytotoxic activity were studied in lytic assays against different cellular targets. Summarized in Table 3 are data demonstrating that the cytotoxicity against Raji cells is readily lost by substitutions at either of two targeted lysines alone but that the relative degree of loss of activity against either erythrocytes or BL-3 cells appears to depend on the amino acid substituted at the respective lysine positions. To better illustrate the relative changes in the phenotypic patterns of these mutants, Fig. 2 shows representative toxin doses versus cytotoxicity curves. Such analyses were performed to generate the percent cytotoxicity levels presented in Table 3, where the listed percent cytotoxicity represents the level of cellular lysis at the highest toxin dose available. At present we are unable to acquire these mutant toxins at higher concentrations and still maintain cytotoxic activity. The percentages of cytotoxicity listed are intended to simplify the discussion for each mutant. The slopes and plateaus of the dose-response curves are more informative, because they reveal the relative changes in mutant toxin efficiency. These data suggest that the retention of a single acylation site results in a form of HlyA that has significant cytotoxicity. From Fig. 2A and B, the estimated toxin doses leading to 50% lysis for sheep erythrocytes and BL-3 cells are the same (0.2 ng). This indicates that although the two cell types have sensitivities similar to that of the wild-type hemolysin, the mutants with putatively only a single acylation can still lyse BL-3 cells but are more dramatically affected in their ability to lyse erythrocytes. The recombinant construction of the $HlyA_{K563C,K689C}$ double substitution results in a mutant toxin which has lost cytotoxic activity against all three cell types examined. In cytotoxicity assays this mutant protein behaves similarly to the $HlyC^-$ pro-HlyA form. The cytotoxicity levels for mutant toxins with alterations outside of the two HlyA acylation sites (e.g., $HlyA_{\Delta 238-259}$ and $HlyA_{\Delta 626-673}$) show that loss of cytotoxicity against one cell type is accompanied by a general loss in cytolytic activity.

The $HlyA_{E684K}$, three $HlyA_{G688}$, and $HlyA_{T694P}$ substitutions each result in loss of D12 reactivity, but their cytotoxic activities are affected differently. For example, the $HlyA_{E684K}$ variant possesses nearly wild-type levels of activity against all three cell types, whereas three different substitutions at $HlyA_{G688}$ yielded mutant toxins with marginal lytic activity against the same cells. The lytic phenotype for $HlyA_{T694P}$ mutant has no detectable erythrolytic or Raji cell cytotoxicity but retains almost 50% of the wild-type BL-3-lytic activity.

The normally nonhemolytic *P. haemolytica* leukotoxin is converted into a hemolytic toxin by substitution of the $HlyA_{K563-H739}$ sequence for the homologous $LktA_{K554-N739}$ region in LktA (9). This HlyA region includes the two acylated lysines. A search for lysines positioned at similar sequences in LktA indicates that the proximal lysine, $LktA_{K554}$, is present, but at the region encompassing the distal site, there is an $LktA_{N684}$ equivalent to $HlyA_{K689}$ (27). No information is available on what sites in LktA are acylated by LktC, although it is known that in an *E. coli* recombinant background, *hlyC* in *trans* to *lktA* will result in

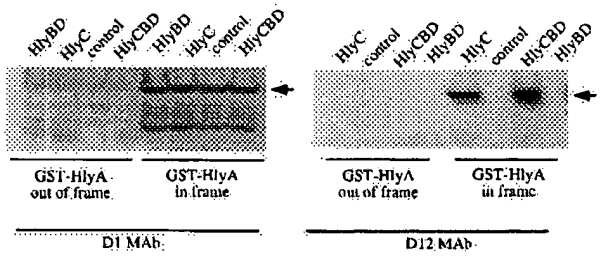


FIG. 1. Immunoblots of cell lysates from different *E. coli* backgrounds containing GST-HlyA gene fusions. Two different GST-HlyA recombinant plasmids were introduced into four different *hly* backgrounds, listed at the tops of the blots. In the left four lanes of each immunoblot are lysates of cells containing a pGEX-2T GST-HlyA recombinant in which there is a +1 frameshift at the GST and $HlyA_{S608-T725}$ junction site. The right four lanes of each blot have lysates from cells containing the gene fusion in frame. Control lanes contain the background strain harboring the vector plasmid pACYC184. The arrow indicates the immunoblot signal representing the appropriately sized fusion peptide for the in-frame gene fusions. The MAb probes used in each blot are listed at the bottom of the figure. The immunoblotting conditions and signal detection are similar to those described previously (25).

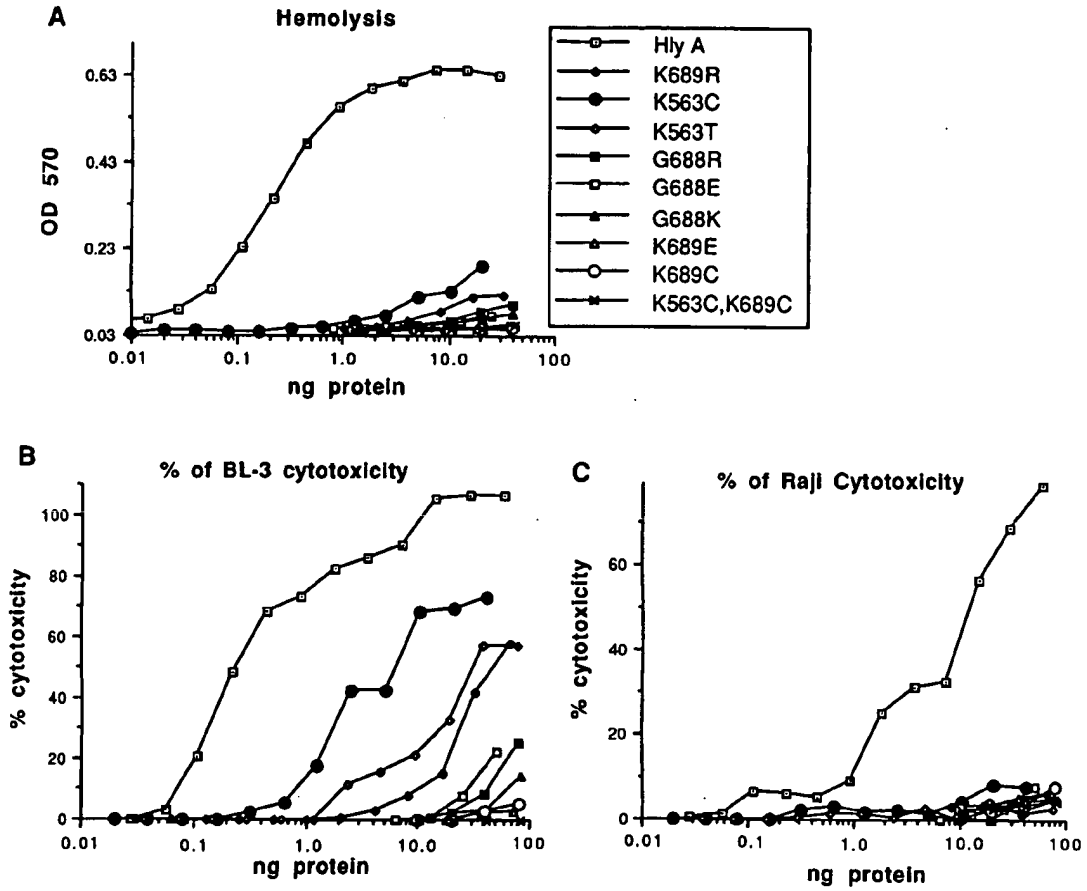


FIG. 2. Dose dependencies of different toxin mutants. Shown are data representative of an experiment in which the amounts of cellular lysis effected by different concentrations of a set of mutant toxins are compared. The conditions for the lysis assays for each of the cell types and the method for measuring toxin concentration are described in the text. (A) Levels of lysis of sheep erythrocytes as monitored by release of $A_{570}$ material versus different toxins. OD 570, optical density at 570 nm. The key to the right indicates the identities of the different HlyA mutants examined in this experiment. Percent cytotoxicity of BL-3 cells (B) and Raji cells (C) versus the same group of mutants described for panel A. The three sets of lysis assays shown were performed on the same day with the same preparations of toxins.

active LktA leukotoxic activity with a target cell specificity similar to that of the wild-type leukotoxin. We hypothesized that a lysine substitution at $LktA_{N684}$ would provide an additional site for LktA acylation and a subsequent change in its cytolytic capabilities. The cytotoxic activities of $LktA_{N684K}$ produced in the *E. coli hylCBD* background were similar to those of the wild-type LktA in the same background (Table 3). Because HlyC acylates HlyA at two lysine positions at least under in vitro conditions (27) and LktA shares only one lysine comparable to those in HlyA, it was inferred that LktA is acylated once by HlyC. Two different substitutions, $LktA_{K554T}$ and $LktA_{K554C}$, were isolated by site-directed mutagenesis to examine the effect of the loss of the putative LktA acylation site. The former mutant protein was preliminarily described in our earlier publication on hybrid toxin construction (9). The $LktA_{K554T}$ substitution was described as a mutant toxin with low but detectable levels of BL-3-lytic activity (~1% of wild-type levels). This *lktA* mutation was subcloned into a new vector background (pBS+) to yield pWAM2157. This LktA mutant along with the new $LktA_{K554C}$ mutant was used in lytic assays which have undergone some recent modifications that enhance their sensitivities (25). At similar doses the $LktA_{K554T}$ and $LktA_{K554C}$ mutants possess 49 and 45% of the wild-type levels of BL-3-lytic activity, respectively. A $LktA_{K554C,N684K}$ double mutant was constructed by subcloning and was tested in the lytic assays. The BL-3-lytic phenotype of this mutant is the same as that of the $LktA_{K554C}$ variant.

The genetic analysis of the HlyA activation sites is well complemented by sequence alignments of these regions among the different RTX toxins. Shown in Fig. 3A and B are alignments of the available RTX toxin amino acid sequences around the lysines described above. The alignment corresponding to the distal HlyA site contains only 6 of the 12 toxin sequences because the residue corresponding to $HlyA_{K689}$ in the other 6 toxin sequences is either an asparagine or a serine, which would not be acylated via the amide linkage by the HlyC apparatus (27). In the comparison of the primary sequences for the two sites (summarized in the Fig. 3C histograms), only glycines preceding the two lysines are strictly conserved. In these two RTX toxin regions, the conserved positions at one site indicated in the Fig. 3C histogram are not matched in their spacing relative to conserved amino acids for the glycine-lysine pair at the other site. Analysis of the predicted HlyA secondary structure by either the Chou-Fasman method (4) or that of Robson and coworkers (10, 24) indicates that the lysines occur at potential bends that may be bracketed by alpha-helixes. When examined on the basis of helical wheel projections of the possible alpha-helices, the conserved, charged residues pre-

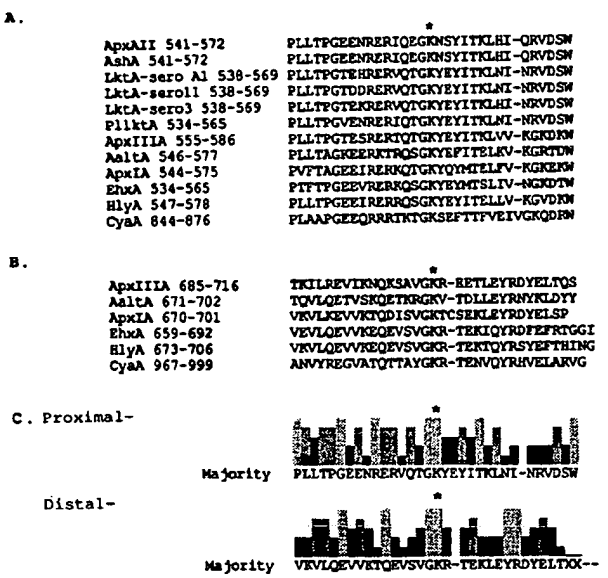


FIG. 3. Amino acid alignments of RTX toxin polypeptide sequences at the regions surrounding the $HlyA_{K563}$ and $HlyA_{K689}$ sites. The sequences of the different RTX toxin polypeptides surrounding the proximal $HlyA_{K563}$ site (A) and the distal $HlyA_{K689}$ site (B) are listed and numbered to the left of the panels. The sequence alignments were performed as previously described (30). (C) Histogram summaries for the amounts of conservation of amino acids for the alignments shown in panels A and B, respectively. *, position of the lysine in each toxin sequence that corresponds to the in vitro-acylated sites in HlyA (27).

ceeding the lysines at either site, although not identical at each site, appear to closely occupy the same longitudinal half of the respective alpha-helices. In the protoxin form, the two modified HlyA regions represent two of the four sequence spans that have the greatest likelihood of being surface exposed by the predicted method of Emini et al. (7). The most likely surface-exposed domain for the prohemolysin is the $HlyA_{500\text{-}520}$ region, which curiously also possesses glycine-lysine amino acid pairs in the RTX toxin alignments.

## DISCUSSION

One of the major discoveries reported here is that *E. coli* hemolysin lytic activities against sheep erythrocytes and Raji cells depend more on the presence of both acylated lysines in HlyA than does its cytotoxic activity against BL-3 cells. The lytic activities of mutant toxins with substitutions at either the $HlyA_{K563}$ or $HlyA_{K689}$ positions are more adversely affected for sheep erythrocytes and Raji cells than for BL-3 cells. This is best exemplified by the $HlyA_{K563T}$ and $HlyA_{K689R}$ mutants, which in dose-response studies have little detectable lytic activity against sheep erythrocytes and Raji cells but which have at least 50% of the wild-type hemolysin BL-3 cytotoxicity levels. The amino acids substituted at these lysines also result in variant toxins with different degrees of loss of the lytic activities. For example, the erythrolytic activity of $HlyA_{K563C}$ is less adversely affected than that of the $HlyA_{K563T}$ mutant. This is also evident for the four substitutions at the $HlyA_{K689}$ site. The $HlyA_{K689R}$ mutant is least affected in its BL-3 cytotoxicity, whereas the $HlyA_{K689E}$ and $HlyA_{K689C}$ mutants are essentially inactive against BL-3 cells. The glycines preceding the acylated lysines are the only residues within the primary sequence of individual RTX toxins that are absolutely conserved in the amino acid sequence alignments presented in Fig. 3. Therefore, we reasoned that substitutions at the $HlyA_{G688}$ site would have alterations in cytolytic activities similar to those substitutions at the neighboring $HlyA_{K689}$ position because they would interfere with the acylation reaction at that site. There are no data available here that indicate that the acylation at $HlyA_{K689}$ is blocked by the $HlyA_{G688}$ substitutions. The loss of the D12 MAb reactivity for these mutant proteins could be related to a glycine contribution to the antibody epitope alone. However, the uniform loss in lytic activities for the $HlyA_{G688}$ mutants, compared with the unequal pattern of loss of lytic activities by the different $HlyA_{K689}$ mutants which can no longer be acylated at position 689, suggests that a glycine residue proximal to the lysine position plays a structural role in the function of the hemolysin larger than just one of recognition by the acylation mechanism. Because the HlyA glycine-lysine pair at position 688 to 689 is predicted to be within a flexible loop immediately distal to a charged alpha-helix, we hypothesize that at the glycine position, free rotation of the polypeptide backbone can occur. This permits potential structural movement of this region of the protein, and such hypothetical movement could occur when the lysine becomes acylated or afterwards when the fatty acid-modified toxin interacts with host cell membranes.

Another important finding described here is the observation that the *P. haemolytica* leukotoxin substitution $LktA_{K554C}$ retains 59% of the lytic activity of LktA activated and secreted in an *E. coli hlyCBD* background. The lysis data for the mutants do not directly address whether the wild-type $LktA_{K554}$ site is acylated in this setting. However, the results do imply that LktA has an alternative acylation site(s) that is used by the HlyC-dependent activation process. The site(s) would have to be different from those identified for either the *E. coli* hemolysin or the *B. pertussis* adenylate cyclase/hemolysin. If a glycine-lysine pair represents the minimum for a consensus site of the RTX HlyC-dependent activation process, then there are nine additional glycine-lysine pairs in the predicted LktA sequence besides the G-553–K-554 pair. The LktA positions of the glycines in these pairs are 429*, 492*, 503*, 526, 668, 753, 772, 781, and 886. The asterisks indicate glycine positions of those pairs which align with similar pairs in HlyA and most other RTX toxins. These three sites also possess charged residues neighboring either side of the glycine-lysine pair and are predicted to be surface exposed regions of LktA. It will be interesting to see by direct biochemical methods where the native and recombinant LktA molecules are modified.

The observation by Hackett et al. (12) that acylation of the *B. pertussis* adenylate cyclase/hemolysin occurs differently in *B. pertussis* and *E. coli* backgrounds with different consequences for hemolytic activity led to our substitution of a lysine at the $LktA_{N684}$ position. The hypothesis was that the availability of a lysine at this LktA site would permit acylation and a gain of erythrolytic function. There is no direct evidence presented here that the $LktA_{N684K}$ mutant protein becomes acylated, but whether or not the proximal $LktA_{K554}$ site is present, the $LktA_{N684K}$ substitution does not have hemolytic activity in an *E. coli* HlyC background. The observation by Hackett et al. combined with the evidence discussed above for the acylation of LktA at a site other than $LktA_{K554}$ raises the possibility that the alternative LktA acylation(s) could inhibit hemolytic activity as does the acylation of the *B. pertussis* adenylate cyclase/hemolysin at $CyaA_{K680}$ in an *E. coli* background (12).

The results with the GST-HlyA gene fusions indicate that in an in vivo setting, a relatively small region of the entire HlyA polypeptide is needed for recognition by the HlyC activation mechanism. The data presented here show that the bulk of the $Ca^{2+}$-binding repeats distal to the $HlyA_{K689}$ site and the ex-

tracellular targeting sequence at the carboxy terminus are not necessary for this process. Additional deletion analysis of the $HlyA_{S608-T725}$ region by the gene fusion approach could further localize the HlyA sequences needed for the modification, but perhaps the more important issues related to this are the structural and functional consequences of the fatty acids added to the HlyA protein. To study these in greater depth, the gene fusion described here represents a new reagent for prospective biochemical and biophysical studies.

In summary, these results continue to support the hypothesis that there are RTX toxin structures that confer different host and cell type-cytotoxic specificities. The activation of the RTX toxins by the RTX HlyC-like gene products appears to play an important role in this intriguing phenotype.

## ACKNOWLEDGMENTS

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DOCUMENT-IDENTIFIER: US 20030118566 A1
TITLE: Compositions and methods for isolation, propagation, and differentiation of human stem cells and uses thereof

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[0182] Animals were sacrificed 60 days after transplantation, brains sectioned and immunostained. Sections were incubated after blocking with 3% BSA and 0.02% Tween 20 for 16 hours at 4.degree. C. All primary antibodies were diluted in PBS containing 3% BSA and 0.02% Tween 20. Antibodies that were used in this study were anti-human nucleus antibodies (1:50, Chemicon) to detect human cells, antitype III .beta.-tubulin antibodies (1:100, Chemicon) to detect neurons, anti tyrosine hydroxylase (1:100 Sigma) and dopa decarboxylase (1:200, Chemicon) antibodies to detect dopaminergic cells, anti gamma amino acid decarboxylase (GAD) antibodies to identify GABAergic neurons (1:1000, Chemicon), anti L-glutamate antibodies to detect glutamatergic neurons (1:50, Signature Immunologics), anti-glycine antibodies to detect glycinergic neurons (1:100, Signature Immunologics), anticholine acetyl transferase (CHAT) to detect cholinergic neurons (1:100 Chemicon) and anti GFAP antibodies (1:500, DAKO) to detect astrocytes. For double staining sections were incubated simultaneously with two primary and secondary antibodies. The second antibodies were goat anti-mouse FITC (1:200, Sigma) and goat anti-rabbit rhodamine (1:200, Boehringer).

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